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(54) **Raffinose synthase genes and their use**

(57) The invention disclosed relates to raffinose synthase genes comprising a nucleotide sequence hybridizable with a nucleotide sequence selected from the group consisting of: (a) a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 1; (b) the nucleotide sequence represented by SEQ ID NO: 2; (c) a nucleotide sequence encoding the amino acid sequence of represented by SEQ ID NO: 3; (d) the nucleotide sequence represented by SEQ ID NO: 4 or by the 236th to 2584th nucleotides in the nucleotide sequence represented by SEQ ID NO: 4; (e) a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 5; (f) the nucleotide sequence represented by SEQ ID NO: 6 or by the 134th to 2467th nucleotides in the nucleotide sequence represented by SEQ ID NO: 6; (g) a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 7; and (h) the nucleotide sequence represented by SEQ ID NO: 8 or by the 1st to 1719th nucleotides in the nucleotide sequence represented by SEQ ID NO: 8; under stringent conditions, and encoding a protein being capable of binding the D-galactosyl group through an  $\alpha$  (1 $\rightarrow$ 6) bond to the hydroxyl group attached to the carbon atom at the 6-position of the D-glucose residue in a sucrose molecule to form raffinose.

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**Description****BACKGROUND OF THE INVENTION**5 **FIELD OF INVENTION**

[0001] The present invention relates to raffinose synthase genes and their use.

**DISCLOSURE OF THE RELATED ART**

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[0002] Raffinose family oligosaccharides are derivatives of sucrose, which are represented by the general formula:  $\alpha$ -D-galactopyranosyl-(1 $\rightarrow$ 6)<sub>n</sub>- $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-fluctofuranoside, and they are called "raffinose" when n is 1, "stachyose" when n is 2, "verbascose" when n is 3, and "ajugose" when n is 4.

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[0003] It has been known that raffinose family oligosaccharides have an effect of giving good conditions of enterobacterial flora, if present at an appropriate amount in food. Therefore, raffinose family oligosaccharides have already been used as a functional food material for addition to some kinds of food and utilized in the field of specific health food. On the other hand, raffinose family oligosaccharides are neither digested nor absorbed in mammals such as human, but are assimilated and decomposed by enterobacteria to generate gases and to cause meteorism and absorption disorder. Therefore, it has been desired to appropriately regulate the amount of raffinose family oligosaccharides in food and feed.

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[0004] Raffinose family oligosaccharides are synthesized by the raffinose family oligosaccharide biosynthesis system beginning with sucrose in many plants. This biosynthesis system normally involves a reaction for the sequential addition of galactosyl groups from galactinol through an  $\alpha$  (1 $\rightarrow$ 6) bond to the hydroxyl group attached to the carbon atom at 6-position of the D-glucose residue in a sucrose molecule. Raffinose synthase is the enzyme concerned in the reaction for producing raffinose by allowing a D-galactosyl group derived from galactinol to form the  $\alpha$  (1 $\rightarrow$ 6) bond with the hydroxyl group attached to the carbon atom at 6-position of the D-glucose residue in a sucrose molecule in the first step of this biosynthesis system. It has been suggested that this enzyme constitutes a rate-limiting step in the above synthesis system, and therefore this enzyme is quite important in the control of biosynthesis of raffinose family oligosaccharides.

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[0005] Then, a method for controlling an expression level or activity of raffinose synthase in plants by utilizing a raffinose synthase gene is effective to control a biosynthesis system of raffinose family oligosaccharides in plants to increase or decrease the production of raffinose in plants. Thus, a raffinose synthase gene which can be used in such a method has been desired.

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**OBJECTS OF THE INVENTION**

[0006] The main object of the present invention is to provide novel raffinose synthase genes from plants.

[0007] This object as well as other objects and advantage of the present invention will become apparent to those skilled in the art from the following description.

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**SUMMARY OF THE INVENTION**

[0008] Under these circumstances, the present inventors have isolated novel genes encoding raffinose synthase from various plants.

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[0009] Thus, the present invention provides a raffinose synthase gene which comprises a nucleotide sequence hybridizable with a nucleotide sequence selected from the group consisting of:

- (a) a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 1,
- (b) the nucleotide sequence represented by SEQ ID NO: 2,
- (c) a nucleotide sequence encoding the amino acid sequence of represented by SEQ ID NO: 3,
- (d) the nucleotide sequence represented by the 236th to 2584th nucleotides in the nucleotide sequence represented by SEQ ID NO: 4,
- (e) a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 5,
- (f) the nucleotide sequence represented by the 134th to 2467th nucleotides in the nucleotide sequence represented by SEQ ID NO: 6,
- (g) a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 7, and
- (h) the nucleotide sequence represented by the 1st to 1719th nucleotides in the nucleotide sequence represented by SEQ ID NO: 8,

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under stringent conditions, and encoding a protein being capable of binding D-galactosyl group through  $\alpha$  (1→6) bond to the hydroxyl group attached to the carbon atom at 6-position of the D-glucose residue in a sucrose molecule to form raffinose.

5 [0010] In a preferred embodiment, the raffinose synthase gene comprises a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 1.

[0011] In another preferred embodiment, the raffinose synthase gene comprises the nucleotide sequence represented by SEQ ID NO: 2.

10 [0012] In another preferred embodiment, the raffinose synthase gene comprises a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 3.

[0013] In another preferred embodiment, the raffinose synthase gene comprises the nucleotide sequence represented by the 236<sup>th</sup> to 2584<sup>th</sup> nucleotides in the nucleotide sequence represented by SEQ ID NO: 4.

[0014] In another preferred embodiment, the raffinose synthase gene comprises a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 5.

15 [0015] In another preferred embodiment, the raffinose synthase gene comprises the nucleotide sequence represented by the 134<sup>th</sup> to 2467<sup>th</sup> nucleotides in the nucleotide sequence represented by SEQ ID NO: 6.

[0016] In another preferred embodiment, the raffinose synthase gene comprises a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 7.

20 [0017] In another preferred embodiment, the raffinose synthase gene comprises the nucleotide sequence represented by the 1<sup>st</sup> to 1719<sup>th</sup> nucleotides in the nucleotide sequence represented by SEQ ID NO: 8.

[0018] In another preferred embodiment, the raffinose synthase gene comprises the nucleotide sequence represented by SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8.

[0019] The invention also relates to a nucleic acid comprising a partial nucleotide sequence of said raffinose synthase genes.

25 [0020] Another embodiment of the invention is a method for detecting a nucleic acid containing a raffinose synthase gene which comprises detecting said nucleic acid by hybridization using said nucleic acids of the invention in a labeled form as a probe.

[0021] Still another embodiment of the invention is a method for amplifying a nucleic acid containing a raffinose synthase gene which comprises amplifying said nucleic acid by polymerase chain reaction (PCR) using said partial nucleic acid as a primer.

30 [0022] The invention also relates to a method for obtaining a raffinose synthase gene which comprises the steps of:

detecting a nucleic acid containing said raffinose synthase gene by hybridization using said labeled nucleic acid as a probe, and

35 recovering the detected nucleic acid.

[0023] In another embodiment the invention relates to a method for obtaining a raffinose synthase gene which comprises the steps of:

40 amplifying a nucleic acid containing said raffinose synthase gene by PCR using said partial nucleotide sequence as a primer, and recovering the amplified nucleic acid.

45 [0024] Furthermore, the present invention relates to nucleic acids comprising said nucleic acids containing the raffinose synthase gene joined to a nucleic acid exhibiting promoter activity in a host cell.

[0025] Another embodiment of the invention are vectors comprising said raffinose synthase gene.

[0026] The invention also relates to transformants, wherein said raffinose synthase gene or said nucleic acid or vector comprising it is introduced into a host cell.

[0027] In a preferred embodiment of the invention said transformed host is a microorganism.

50 [0028] In another preferred embodiment said transformed host is a plant.

[0029] Furthermore, the present invention relates to a method for producing a raffinose synthase which comprises the steps of:

55 culturing or growing a transformant of the present invention to produce the raffinose synthase, and collecting the raffinose synthase.

[0030] Finally, the present invention relates to raffinose synthases encoded by the nucleic acids of the present invention.

[0031] The term "nucleic acid" used herein means an oligomer compound or a high molecular compound which is generally called "DNA" or "RNA".

# DETAILED DESCRIPTION OF THE INVENTION

[0032] The gene engineering techniques described below can be carried out, for example, according to methods described in "Molecular Cloning; A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press, ISBN 0-87969-309-6; "Current Protocols In Molecular Biology" (1987), John Wiley & Sons, Inc. ISBN 0-471-50338-X; "Current Protocols In Protein Science" (1995), John Wiley & Sons, Inc. ISBN 0-471-11184-8.

[0033] The genes of the present invention can be obtained from soybean, plants belonging to the families Chenopodiaceae such as beet, etc. and Cruciferae such as mustard, rapeseed, etc. Specific examples of the genes of the present invention include those comprising a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 1, the nucleotide sequence represented by SEQ ID NO: 2, a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 3, the nucleotide sequence represented by SEQ ID NO: 4 or by the 236th to 2584th nucleotides in the nucleotide sequence represented by SEQ ID NO: 4, a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 5, the nucleotide sequence represented by SEQ ID NO: 6 or by the 134th to 2467th nucleotides in the nucleotide sequence represented by SEQ ID NO: 6, a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 7, the nucleotide sequence represented by SEQ ID NO: 8 or by the 1st to 1719th nucleotides in the nucleotide sequence represented by SEQ ID NO: 8, and the like.

[0034] The genes of the present invention can be obtained, for example, by the following method.

[0035] That is, the genes of the present invention derived from soybean can be obtained, for example, by the following method.

[0036] For example, the gene can be obtained by a hybridization method using a nucleic acid having the nucleotide sequence represented by SEQ ID NO: 2 or its partial nucleotide sequence as a probe to detect a nucleic acid fragment which hybridizes to the probe in DNAs derived from soybean, followed by isolating the detected nucleic acid.

[0037] In this method, first, a nucleic acid to be used as the probe is prepared. As such a nucleic acid, for example, there is a nucleic acid composed of an oligonucleotide chemically synthesized by a conventional method on the basis of the nucleotide sequence of SEQ ID NO: 2. Specific example thereof includes a nucleic acid having the 800th to the 899th nucleotides in the nucleotide sequence represented by SEQ ID NO: 2.

[0038] Alternatively, the gene of the present invention derived from soybean can be obtained by the following method.

[0039] For example, tissue of soybean (*Glycine max*) is frozen in liquid nitrogen and ground physically with a mortar or other means into finely divided tissue debris powder. From the tissue debris powder, RNA is extracted by a conventional method. A commercially available RNA extraction kit can be utilized in the extraction. RNA is recovered from thus-obtained RNA extract by ethanol precipitation. Poly-A tailed RNA is fractionated from thus-recovered RNA by a conventional method. A commercially available oligo-dT column can be utilized in this fractionation. cDNA is synthesized from the poly-A tailed RNA thus obtained by a conventional method. The synthesis can be carried out by using a commercially available cDNA synthesis kit. DNA is amplified by PCR using the above-obtained cDNA as the template and primers designed and synthesized on the basis of the nucleotide sequence of SEQ ID NO: 2. More specifically, as the primers, for example, there are primers 11 and 12 shown in List 1 hereinafter. When PCR is carried out by using these primers and as the template cDNA derived from soybean, the genes of the present invention derived from soybean, .g., the "raffinose synthase gene having a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 1" and the "raffinose synthase gene having the nucleotide sequence of SEQ ID No: 2" can be obtained.

[0040] The amplified DNA can be cloned according to a conventional method, for example, described in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press; or "Current Protocols In Molecular Biology" (1987), John Wiley & Sons, Inc. ISBN 0-471-50338-X. Alternatively, cloning can be carried out, for example, by using a commercially available cloning kit such as TA cloning kit (Invitrogen) and a commercially available plasmid vector such as pBluescript II (Stratagene). The nucleotide sequence of the DNA clone can be determined by dideoxy terminating method such as that described by F. Sanger, S. Nicklen, A.R. Coulson, Proceedings of National Academy of Science U.S.A. (1977), 74, pp. 5463-5467. For example, preferably, a commercially available kit such as ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit manufactured by Perkin-Elmer can be used.

List 1

List 1		
Primer 11:	ccaatctgat catgcttggt ccgaa	25mer
Primer 12:	ggaacaaagt tatgcactat tatttaaggt	30mer

[0041] The genes of the present invention derived from a Chenopodiaceae plant such as beet can be obtained by the following method.

[0042] For example, tissue of a Chenopodiaceae plant such as beet (*Beta vulgaris*) is frozen in liquid nitrogen and ground physically with a mortar or other means into finely divided tissue debris powder. From this tissue debris powder, RNA is extracted by a conventional method. A commercially available RNA extraction kit can be utilized in the extraction. RNA is recovered from the thus-obtained RNA extract by ethanol precipitation. From the recovered RNA, poly-A tailed RNA is fractionated by a conventional method. A commercially available oligo-dT column can be utilized in this fractionation. cDNA is synthesized from the poly-A tailed RNA thus obtained by a conventional method. The synthesis can be carried out by utilizing a commercially available cDNA synthesis kit. DNA is amplified by PCR using the above-obtained cDNA as the template and primers designed and chemically synthesized on the basis of the nucleotide sequence of SEQ ID NO: 4. More specifically, as the primers, for example, there are primers 21 and 22 shown in List 2 hereinafter. When PCR is carried out by using these primers and as the template cDNA derived from beet, the genes of the present invention derived from beet, e.g., the "raffinose synthase gene having a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 3," and the "raffinose synthase gene having a nucleotide sequence of SEQ ID NO: 4" can be obtained. According to a particular purpose, the PCR primers can also be designed and synthesized on the basis of the nucleotide sequence of SEQ ID NO: 4. For example, in order to amplify the "raffinose synthase gene having the nucleotide sequence represented by the 236th to the 2584th nucleotides in the nucleotide sequence represented by SEQ ID NO: 4", preferably, oligonucleotides having the nucleotide sequences represented by primers 23 and 24 in List 2 below are synthesized and used as the primers.

[0043] The amplified DNA can be cloned according to a conventional method, for example, described in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press; or "Current Protocols In Molecular Biology" (1987), John Wiley & Sons, Inc. ISBN 0-471-50338-X. Alternatively, cloning can be carried out by using a commercially available cloning kit such as TA cloning kit (Invitrogen) and a commercially available plasmid vector such as pBluescript II (Stratagene). The nucleotide sequence of the DNA clone can be determined, for example, by dideoxy terminating method such as that described by F. Sanger, S. Nicklen, A.R. Coulson, Proceedings of National Academy of Science U.S.A. (1977), 74, pp. 5463-5467. For example, preferably, a commercially available kit such as ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit manufactured by Perkin-Elmer can be used.

List 2

List 2		
Primer 21;	ctaccaaatt ccacaactta aagtca	27mer
Primer 22:	ggaataataa ggtcacaca tactgtactc tc	32mer
Primer 23:	atggctcaa gcttagcaa ggaaaattcc	30mer
Primer 24:	tcaaataag tactcaacag tggtaaaacc	30mer

[0044] The genes of the present invention derived from Cruciferae plants such as mustard (*Brassica juncea*) and rapeseed (*Brassica napus*) can be obtained by the following method.

[0045] For example, tissue of a Cruciferae plant such as mustard or rapeseed is frozen in liquid nitrogen and ground physically with a mortar or other means into finely divided tissue debris powder. From the tissue debris powder, RNA is extracted by a conventional method. A commercially available RNA extraction kit can be utilized in the extraction. The RNA is recovered from thus-obtained RNA extract by ethanol precipitation. Poly-A tailed RNA is fractionated from the RNA thus recovered by a conventional method. A commercially available oligo-dT column can be utilized in the fractionation. cDNA is synthesized from the poly-A tailed RNA thus obtained by a conventional method. The synthesis can be carried out by using a commercially available cDNA synthesis kit. DNA are amplified by PCR using the above-obtained cDNA as a template and primers designed and chemically synthesized on the basis of the nucleotide sequence of SEQ ID NO: 6. For example, when PCR is carried out by using cDNA derived from mustard (*Brassica juncea*) as the template and primers 33 and 34 shown in List 3 hereinafter, the genes from Cruciferae plants of the present invention, e.g., the "raffinose synthase gene having a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 5," and the "raffinose synthase gene having the nucleotide sequence represented by the 1st to 2654th nucleotides in the nucleotide sequence represented by SEQ ID NO: 6" can be obtained. According to a particular purpose, the PCR primers can also be designed and synthesized on the basis of the nucleotide sequence of SEQ ID NO: 6. For example, in order to amplify DNA encoding the open reading frame region of the "raffinose synthase gene having a nucleotide sequence encoding a protein having the amino acid sequence of SEQ ID NO: 5", and the "raffinose synthase gene having the nucleotide sequence represented by the 134th to the 2467th nucleotides of SEQ ID NO: 6", preferably, oligonucleotides having the

nucleotide sequences represented by primers 35 and 36 in List 3 are synthesized and used as the primers.

[0046] The amplified DNA can be cloned according to a conventional method, for example, described in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press; or "Current Protocols In Molecular Biology" (1987), John Wiley & Sons, Inc. ISBN 0-471-50338-X. Alternatively, cloning can be carried out, for example, by using a commercially available TA cloning kit (Invitrogen) or a commercially available plasmid vector such as pBluescript II (Stratagene). The nucleotide sequence of the DNA clone can be determined by dideoxy terminating method such as that described by F. Sanger, S. Nicklen, A.R. Coulson, Proceedings of National Academy of Science U.S.A. (1977), 74, pp. 5463-5467. For example, preferably, the commercially available ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit of Perkin-Elmer can be used.

List 3		
Primer 31:	ttggaagaga agacgccgcc ggaatcgtc	30mer
Primer 32:	ttaagcccg gcgagagctc tggccggaca	30mer
Primer 33:	accaatcaa aatctcatca aataatcgca	30mer
Primer 34:	aaataatagg ggagtagcaa attacaccac	30mer
Primer 35:	atggctccac cgagcgtaat taaatccga	29mer
Primer 36:	ctaaaactca tacttaatat aagacaaacc	30mer

[0047] Then, a nucleic acid having a partial nucleotide sequence of the gene of the present invention (hereinafter referred to as "the gene fragment") which is obtained by the above-described method is labeled and then used as a probe in a hybridization method. The probe can be hybridized to, for example, DNA derived from soybean, a Chenopodiaceae plant or a Cruciferae plant to detect a nucleic acid having the probe specifically bound thereto, thereby detecting a nucleic acid having the raffinose synthase gene.

[0048] As the DNA derived from soybean, a Chenopodiaceae plant such as beet or a Cruciferae plant such as mustard or rapeseed, for example, a cDNA library or a genomic DNA library of these plants can be used. The gene library may also be a commercially available gene library as such or a library constructed according to a conventional library construction method, for example, described in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press; "Current Protocols In Molecular Biology" (1987), John Wiley & Sons, Inc. ISBN 0-471-50338-X.

[0049] As the hybridization method, for example, plaque hybridization or colony hybridization can be employed, and they are selected depending upon the kind of vector used in the construction of a library. More specifically, when the library to be used is constructed with a phage vector, a suitable host microorganism is mixed with the phage of the library under infectious conditions to obtain transformants. The transformant is further mixed with a soft agar medium, and the mixture is plated on an agar medium. Thereafter, the mixture is cultured at 37°C until a plaque of an appropriate size appears. When the library to be used is constructed with a plasmid vector, the plasmid is introduced into a suitable host microorganism to form transformants. The transformant obtained is diluted to a suitable concentration and the dilution is plated on an agar medium, after which it is cultured at 37°C until a colony of an appropriate size appears. In either case of the above libraries, a membrane filter is placed on the surface of the agar medium after the above cultivation, so that the phage or transformant is transferred to the membrane. This membrane is denatured with an alkali, followed by neutralization, and for example, when a nylon membrane is used, the membrane is irradiated with ultraviolet light, so that DNA of the phage or transformant is fixed on the membrane. This membrane is then subjected to a hybridization method wherein the gene fragment which has a partial nucleotide sequence of the gene of the present invention and labeled by a conventional method (hereinafter referred to as "the labeled gene fragment") is used as a probe. For this method, reference may be made, for example, to D.M. Glover ed., "DNA cloning, a practical approach" IRL PRESS (1985), ISBN 0-947946-18-7. There are various reagents and temperature conditions to be used in the hybridization. For example, in general, prehybridization is carried out by immersion of the membrane in a prehybridization solution [6 x SSC (0.9 M NaCl, 0.09 M citric acid), 0.1 to 1 (w/v)% SDS, 100 µg/ml denatured salmon sperm DNA] and incubation at 65°C for 1 hour. Then, hybridization is carried out by addition and mixing of the labeled gene fragment thereto and incubating the membrane at 42 to 68°C for 4 to 16 hours.

[0050] In the present invention, the "stringent conditions" are those wherein incubation is carried out, for example, at 65 to 68°C in the above hybridization.

[0051] After hybridization, the membrane is taken out and is washed with 2 x SSC containing 0.1 to 1 (w/v)% SDS,

further rinsed with 0.2 x SSC containing 0.1 to 1 (w/v)% SDS, and then dried. The membrane is analyzed, for example, by autoradiography or other techniques to detect the position of the probe on the membrane, thereby detecting the position on the membrane of a nucleic acid having a nucleotide sequence homologous to that of the probe used. The clone corresponding to the position of the nucleic acid thus detected on the membrane is identified in the original agar medium and the positive clone is selected so that the clone having the nucleic acid can be isolated. The same procedures of detection are repeated to purify the clone having the nucleic acid.

[0052] Alternatively, a commercially available kit such as GENE TRAPPER cDNA Positive Selection System kit (GibcoBRL) can be used. In this method, first, a single-stranded DNA library is hybridized with the biotinylated gene fragment (i.e., probe), followed by adding streptavidin-bound magnet beads and mixing. From the mixture, the streptavidin-bound magnetic beads are collected with a magnet, so that single-stranded DNA having a nucleotide sequence homologous to that of the probe used, which has been bound to these beads through the gene fragment, biotin and streptavidin, is collected and detected. The single-stranded DNA collected can be converted into a double-strand form by reaction with a suitable DNA polymerase using a suitable oligonucleotide as a primer.

[0053] As described above, a nucleic acid containing raffinose synthase gene can be obtained by detecting a nucleic acid hybridizable to the gene fragment in DNAs of a gene library derived from soybean, a Chenopodiaceae plant or a Cruciferae plant, purifying a clone having the nucleic acid and isolating phage or plasmid DNA from the clone. By preparing the restriction map or determining the nucleotide sequence of the nucleic acid thus obtained according to a conventional method, the nucleic acid containing the gene of the present invention can be confirmed.

[0054] For example, the gene of the present invention from a Chenopodiaceae plant can be confirmed by the following point;

[0055] The amino acid encoded by the nucleotide sequence thus determined has 75% or more homology to the amino acid sequence represented by the 103rd to 208th amino acids in the amino acid sequence of SEQ ID NO: 3;

80% or more homology to the amino acid sequence represented by the 255th to 271st amino acids in the amino acid sequence of SEQ ID NO:3;

70% or more homology to the amino acid sequence represented by the 289th to 326th amino acids in the amino acid sequence of SEQ ID NO: 3; or

70% or more homology to the amino acid sequence represented by the 610th to 696th amino acids in the amino acid sequence of SEQ ID NO: 3.

[0056] The gene of the present invention from a Cruciferae plant can be confirmed, for example, by the following point;

[0057] The amino acid sequence encoded by the nucleotide sequence determined has 75% or more homology to the amino acid sequence represented by the 111th to 213th amino acids in the amino acid sequence of SEQ ID NO: 5;

80% or more homology to the amino acid sequence represented by the 260th to 275th amino acids in the amino acid sequence of SEQ ID NO: 5;

70% or more homology to the amino acid sequence represented by the 293rd to 325th amino acids in the amino acid sequence of SEQ ID NO: 5; or

70% or more homology to the amino acid sequence represented by the 609th to 695th amino acids in the amino acid sequence of SEQ ID NO: 5.

[0058] The "homology" used herein means the proportion of the number of amino acids in a region, which are identical to those in a different region to be compared, to the number of the entire amino acids in the former region, upon comparing regions having similarity in two amino acid sequences. In this respect, it is preferred that the region having similarity contains more amino acids. Such homology of amino acid sequences can be evaluated by using a commercially available gene analysis software such as GENETIX (Software Kaihatu K.K.).

[0059] Further, according to the same manner as described above, a nucleic acid containing raffinose synthase gene can be detected by hybridization to DNA from the desired organism using the gene fragment as a probe to detect a nucleic acid to which the probe specifically binds (hereinafter referred to as the detection method of the present invention). The gene fragment used herein can be chemically synthesized according to a conventional method on the basis of the nucleotide sequence represented by SEQ ID NO: 2, 4, 6 or 8. Alternatively, it can be prepared by PCR using as primers oligonucleotides chemically synthesized according to a conventional method on the basis of the nucleotide sequence represented by SEQ ID NO: 2, 4, 6 or 8.

[0060] The gene fragment may be a part of the non-translated region of the raffinose synthase gene as well as the open reading frame thereof. For example, an oligonucleotide having the same nucleotide sequence as a part of that of 5'-upstream side such as the 1st to 235th nucleotides in the nucleotide sequence of SEQ ID NO: 4, the 1st to 133rd nucleotides in the nucleotide sequence of SEQ ID NO: 6 and the like, or a part of that of 3'-downstream side such as the 2588th to 2675th nucleotides in the nucleotide sequence of SEQ ID NO: 4, the 2468th to 2676th nucleotides in the

nucleotide sequence of SEQ ID NO: 6 and the like.

[0061] When PCR is carried out by using the gene fragment as primers, it is possible to amplify a nucleic acid containing raffinose synthase gene from DNA derived from the desired organism (hereinafter referred to as the amplification method of the present invention).

[0062] More specifically, for example, oligonucleotides having the nucleotide sequences of the gene fragment are designed and chemically synthesized according to a conventional method. In general, it is preferred that the number of nucleotides is more from a viewpoint that the specificity of annealing is ensured. It is, however, also preferred that the number of nucleotides is not so many from viewpoints that the primers themselves are liable to have a higher structure giving possible deterioration of the annealing efficiency and that complicated procedures are required in the purification after the synthesis. Normally, oligonucleotides composed of 15 to 50 bases are preferred. In this respect, based on the codon table showing the correspondence of amino acids encoded by codons, a mixture of primers can also be synthesized by using a mixture of plural bases so that a residue at a specified position in a primer is changed to different bases according to the variation of codons which can encode one certain amino acid. Alternatively, for example, a base such as inosine which can form a base pair with plural bases can be used instead of the above mixture of plural bases.

#### Coding Table

Phe: UUU, UUC	Ser: UCU, UCC, UCA, UCG, AGU, AGC
Tyr: UAU, UAC	Cys: UGU, UGC
Stop: UAA, UAG, UGA	Trp: UGG
Leu: UUA, UUG, CUU, CUC, CUA, CUG	Pro: CCU, CCC, CCA, CCG
His: CAU, CAC	Gln: CAA, CAG
CGC, CGA, CGG, AGA, AGG	Arg: CGU, CGC, CGA, CGG, AGA, AGG
Ile: AUU, AUC, AUA	Thr: ACU, ACC, ACA, ACG
Asn: AAU, AAC	Lys: AAA, AAG
Met: AUG	
Val: GUU, GUC, GUA, GUG	Ala: GCU, GCC, GCA, GCG
Asp: GAU, GAC	Gly: GGU, GGC, GGA, GGG
Glu: GAA, GAG	

[0063] In the above codon table, each codon is shown as the nucleotide sequence in mRNA and its right hand is the 5'-terminus. U represents uracil base in RNA and corresponds to thymine base in DNA.

[0064] An oligonucleotide having the same nucleotide sequence as the coding strand of the double-stranded DNA of the gene of the present invention is called a "sense primer" and that having a nucleotide sequence complementary to the coding strand is called an "antisense primer".

[0065] A sense primer having the same nucleotide sequence as that of 5'-upstream side in the coding strand of the gene of the present invention, and an antisense primer having a nucleotide sequence complementary to the nucleotide sequence on the 3'-downstream side in this coding strand are used in combination for PCR reaction, for example, with a gene library, genomic DNA or cDNA as the template to amplify DNA. As the gene library to be used, for example, there are a cDNA library and a genomic library derived from soybean, a Chenopodiaceae plant such as beet or a Cruciferae plant such as mustard or rapeseed, etc. The gene library may also be a library constructed according to a conventional library construction method, for example, described in "Molecular Cloning; A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press; "Current Protocols In Molecular Biology" (1987), John Wiley & Sons, Inc.



ISBN 0-471-50338-X, or a commercially available gene library as such. As the genomic DNA or cDNA, for example, there are those prepared from soybean, a Chenopodiaceae plant such as beet or a Cruciferae plant such as mustard or rapeseed, etc. For example, PCR is carried out by using the primers 31 and 32 in the above List 3 and as the template cDNA derived from mustard to amplify DNA having the nucleotide sequence represented by the 749th to 1215th nucleotides in the nucleotide sequence of SEQ ID NO: 6. Further, PCR is carried out by using the primers and as the template cDNA derived from rapeseed to amplify DNA having the nucleotide sequence represented by the 1st to 467th nucleotides in the nucleotide sequence of SEQ ID NO: 8. The nucleic acid thus amplified can be confirmed by conventional electrophoresis. The nucleic acid can be cloned according to a conventional method such as that described in "Molecular Cloning; A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press or "Current Protocols in Molecular Biology" (1987), John Wiley & Sons, Inc., ISBN 0-471-50338-X. For the nucleic acid, its restriction map is prepared or its nucleotide sequence is determined by a conventional method, so that the nucleic acid containing raffinose synthase gene or a part thereof can be identified. When the nucleic acid contains a part of raffinose synthase, PCR can be carried out on the basis of its nucleotide sequence to amplify the nucleic acid containing the 5'-upstream side nucleotide sequence or the 3'-downstream side nucleotide sequence. That is, based on the nucleotide sequence of the above-obtained nucleic acid, an antisense primer is designed and synthesized for amplification of the 5'-upstream side part, and a sense primer is designed and synthesized for amplification of the 3'-downstream side part. The nucleotide sequence of the 5'-upstream side part or 3'-downstream side part of the nucleotide sequence already obtained can be determined by the RACE method using these primers and a commercially available kit such as Marathon Kit of Clontech. The full length raffinose synthase gene can be obtained by synthesizing new primers based on both terminal sequences in the nucleotide sequence thus determined and carrying out PCR again.

[0066] The above detection method of the present invention can also be used in the analysis of genotypes of a plant such as soybean, a Chenopodiaceae plant or a Cruciferae plant, etc. More specifically, for example, a genomic DNA derived from soybean, a Chenopodiaceae plant or a Cruciferae plant is prepared according to a conventional method, for example, described in "Cloning and Sequence (Plant Biotechnology Experiment Manual)" compiled under the supervision of Itaru Watanabe, edited by Masahiro Sugiura, published by Noson Bunka-sha, Tokyo (1989). The genomic DNA is digested with at least several kinds of restriction enzymes, followed by electrophoresis. The electrophoresed DNA is blotted on a filter according to a conventional method. This filter is subjected to hybridization with a probe prepared from DNA having the gene fragment by a conventional method, and DNA to which the probe hybridizes is detected. The DNAs detected are compared in length between different varieties of a specified plant species. The differences in length make possible the analysis of differences in phenotypic characteristics accompanied with the expression of raffinose family oligosaccharides between these varieties. Furthermore, when the DNAs detected by the above method are compared in length between the gene recombinant plant and the non-gene recombinant plant of the same variety, the former plant can be distinguished from the latter plant by the detection of hybridizing bands greater in number or higher in concentration for the former plant than for the latter plant. This method can be carried out according to the RFLP (restriction fragment length polymorphism) method, for example, described in "Plant PCR Experiment Protocols" compiled under the supervision of Ko Shimamoto and Takuji Sasaki, published by Shujun-sha, Tokyo (1995), ISBN 4-87962-144-7, pp. 90-94.

[0067] Further, the amplification method of the present invention can be used for an analysis of genes of soybean, a Chenopodiaceae plant or a Cruciferae plant, etc. More specifically, for example, the amplification method of the present invention is carried out by using plant genomic DNA prepared from soybean, a Chenopodiaceae plant or a Cruciferae plant to amplify DNA. The amplified DNA is mixed with a formaldehyde solution, followed by heat denaturing at 85°C for 5 minutes and then quickly cooling on ice. A sample thereof is subjected to electrophoresis on, for example, 6 (w/v)% polyacrylamide gel containing 0 (v/v) % or 10 (v/v) % of glycerol. For this electrophoresis, a commercially available electrophoresis apparatus such as that for SSCP (Single Strand Conformation Polymorphism) can be used and the electrophoresis can be carried out with maintaining the gel at a constant temperature, for example, at 5°C, 25°C, 37°C, etc. From the electrophoresed gel, DNA is detected, for example, by a method such as silver staining method with a commercially available reagent. From the differences of behavior between the varieties in the electrophoresis of the DNA detected, a mutation in the raffinose synthase gene is detected, and an analysis is carried out for differences caused by the mutation in phenotypic characteristics accompanied with the expression of raffinose family oligosaccharides. This method can be carried out according to the SSCP method, for example, described in "Plant PCR Experiment Protocols" compiled under the supervision of Ko Shimamoto and Takuji Sasaki, published by Shujun-sha, Tokyo (1995), ISBN 4-87962-144-7, pp. 141-146.

[0068] The analysis of the plant gene from soybean, a Chenopodiaceae plant or a Cruciferae plant by the above detection method or amplification method of the present invention can be used not only for the analysis of differences in phenotypic characteristics accompanied with the expression of raffinose family oligosaccharides, but also, for example, for the selection of clones having the desired characters upon production of a novel variety of soybean, a Chenopodiaceae plant or a Cruciferae plant. Further, it can also be used for identification of a clone thus produced and having the characters derived from a recombinant plant upon producing a plant variety using the recombinant plant.

[0069] For expression of the gene of the present invention in cells of a host, preferably, a nucleic acid comprising a nucleic acid fragment which contains the gene of the present invention, and a nucleic acid fragment which has a promoter activity in the host cells and joined to the former nucleic acid fragment (hereinafter referred to as the expression nucleic acid of the present invention) can be used.

[0070] The nucleic acid fragment having promoter activity in the expression nucleic acid of the present invention is not limited to a specific one, so long as it is functionable in a host to be transformed. For example, there are synthetic promoters functionable in *Escherichia coli*, such as *E. coli* lactose operon promoter, *E. coli* tryptophan operon promoter and tac promoter, etc.; yeast alcohol dehydrogenase gene (ADH) promoter, adenovirus major late (Ad.ML) promoter, SV40 early promoter, baculovirus promoter and the like. When the host is a plant, the promoter may include, for example, T-DNA derived constitutive promoters such as nopaline synthase gene (NOS) promoter, octopine synthase gene (OCS) promoter, etc.; plant virus-derived promoters such as cauliflower mosaic virus (CaMV)-derived 19S and 35S promoters; inducible promoters such as phenylalanine ammonia-lyase (PAL) gene promoter, chalcone synthase (CHS) gene promoter, pathogenesis-related protein (PR) gene promoter, etc. Furthermore, vector pSUM-GY1 (see JP-A 06-189777/1994) can also be used, which has a promoter giving specific expression in a specified plant tissue, e.g., a promoter of soybean-derived seed storage protein glycinin gene (JP-A 6-189777).

[0071] Furthermore, a nucleic acid fragment having a terminator activity can be joined to the expression nucleic acid of the present invention. In this case, it is generally preferred that the expression nucleic acid of the present invention is constructed so that the nucleic acid fragment having a terminator activity is positioned downstream the raffinose synthase gene. The terminator to be used is not particularly limited, so long as it is functionable in cells of a host to be transformed. For example, when the host is a plant, there are T-DNA derived constitutive terminators such as nopaline synthase gene (NOS) terminator, etc.; plant derived terminators such as terminators of allium virus GV1 or GV2, and the like.

[0072] The expression nucleic acid of the present invention can be introduced into a host cell according to a conventional gene engineering technique to obtain a transformant. If necessary, the expression nucleic acid of the present invention can be inserted into a vector having a suitable marker depending upon a particular transformation technique for introduction of the nucleic acid into a host cell.

[0073] A vector into which the expression nucleic acid of the present invention is inserted can be introduced into a microorganism according to a conventional method, for example, described in "Molecular Cloning; A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor laboratory Press or "Current Protocols in Molecular Biology" (1987), John Wiley & Sons, Inc., ISBN 0-471-50338-X. The microorganism transformed with the vector can be selected on the basis of a selection marker such as antibiotic resistance, auxotrophy or the like. In case that the gene of the present invention is joined to the downstream of an inducible promoter (e.g., tac promoter) in the translatable form in the selected microorganism (e.g., *E. coli* transformant), a translated product of the gene of the present invention can be expressed under conventional culture and inducible conditions and can be recovered as a peptide or a protein.

[0074] The raffinose synthase activity of the translated product of the gene of the present invention thus prepared can be measured by, for example, a method described in L. Lehle and W. Tanner, Eur. J. Biochem., 38, 103-110 (1973) to identify the translated product having the "capability of binding D-galactosyl group through  $\alpha$  (1 $\rightarrow$ 6) bond to the hydroxyl group attached to the carbon atom at 6-position of the D-glucose residue in sucrose molecule". More specifically, for example, the gene of the present invention is cloned in pGEX-4T3 (Pharmacia) to obtain a plasmid containing the expression nucleic acid of the present invention. The resultant plasmid is introduced into, for example, *E. coli* HB101 strain to obtain a transformant. The resultant transformant is cultured overnight and 1 ml of the culture is inoculated into 100 ml of LB culture medium. It is incubated at 37°C for about 3 hours and IPTG (isopropylthio- $\beta$ -D-galactoside) is added at a final concentration of 1 mM, followed by further incubation for 5 hours. Cells are recovered from the culture broth by centrifugation and are suspended by addition of 10 times of the cell weight of 100 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM DTT (dithiothreitol), 1 mM PMSF (phenylmethylsulfonyl fluoride) and 1 mM benzamide. The suspension is sonicated with an ultrasonic disrupter (Branson) to disrupt the cells. The disrupted cell suspension is centrifuged to recover a soluble protein solution. The resultant protein solution is added to a reaction mixture containing at final concentrations of 100 mM Tris-HCl (pH 7.4), 5 mM DTT (dithiothreitol), 0.01% BSA, 200  $\mu$ M sucrose, 5 mM galactinol and 31.7  $\mu$ M [ $^{14}$ C] sucrose. The reaction mixture is incubated at 37°C, followed by addition of 1.5 times in volume of ethanol and stirring. Insoluble materials are removed by centrifugation, the supernatant is spotted on, for example, a HPTLC cellulose thin layer chromatography plate (Merck, HPTLC plates cellulose) and then the plate is developed with n-butanol-pyridine-water-acetic acid (60 : 40 : 30 : 3). The developed plate is dried and analyzed with an imaging analyzer (FUJIX Bio-Image Analyzer BAS-2000II manufactured by Fuji Film) to determine [ $^{14}$ C] raffinose produced to measure the raffinose synthase activity.

[0075] In addition, the translated product as prepared above can also be used as an antigen for producing an antibody. The antibody thus produced can be used for, for example, detection and determination of the gene of the present invention in a crude protein extract prepared from an organism such as a plant.

[0076] When the host is a plant, the vector into which the gene of the present invention is inserted can be introduced

into plant cells by a conventional means such as *Agrobacterium* infection method (JP-B 2-58917 and JP-A 60-70080), electroporation into protoplasts (JP-A 60-251887 and JP-B 5-68575) or particle gun method (JP-A 5-508316 and JP-A 63-258525). The plant cell transformed by the introduction of the vector can be selected on the basis of a selection marker, for example, resistance to an antibiotic such as kanamycin or hygromycin. From the plant cell thus transformed, a transformed plant can be regenerated by a conventional plant cell cultivation method, for example, described in "Plant Gen Manipulation Manual (How to Produce Transgenic Plants)" written by Uchimiya, 1990, Kodansha Scientific (ISBN 4-06-153513-7), pp. 27-55. Furthermore, the collection of seeds from the transformed plant also makes it possible to proliferate the transformed plant. In addition, crossing between the transformed plant obtained and the non-transformed plant makes it possible to produce progenic plants with the characters of the transformed plant.

[0077] As gene engineering techniques in soybean, basically, the above general techniques can be employed. More specifically, "transformation of soybean plant strain by particle gun" described in EP 301749, gene introduction methods, for example, described in Torisky, R.S., Kovacs, L., Avdiushko, S., Newman, J.D., Hunt, A.G. and Collins, G.B., "Development of a binary vector system for plant transformation based on the supervirulent *Agrobacterium tumefaciens* strain Chry5", Plant Cell Rep., (1997), 17, p. 102-108, etc. can be employed.

[0078] As gene engineering techniques in a Chenopodiaceae plant, basically, the above general techniques can be employed. More specifically, gene introduction methods, for example, described in M. Mannerlof, S. Turesson, P. Steen and P. Tenning, "Transgenic sugar beet tolerant to glyphosate", Euphytica (1997), 94, p. 83-91, B.K. Konwar, "Agrobacterium tumefaciens-Mediated Genetic Transformation of Sugar Beet (*Beta vulgaris* L.)", J. Plant Biochemistry & Biotechnology (1994), 3, p. 37-41 can be employed.

[0079] As gene engineering techniques in a Cruciferae plant, basically, the above general techniques can be employed. More specifically, the gene introduction can be carried out according to a method, for example, described in J. Fry, A. Barnason and R.B. Horsch, "Transformation of Brassica napus with *Agrobacterium tumefaciens* based vectors", Plant Cell Reports (1987), 6, 321-325.

[0080] For example, when gene introduction is carried out by *Agrobacterium* infection method, first, the above-described expression nucleic acid of the present invention is inserted into a binary vector. The resultant vector can be introduced into, for example, *Agrobacterium tumefaciens* LBA 4404 strain which has been converted into a competent state by treatment with calcium chloride. A transformant can be selected by an appropriate selection method according to the selection marker gene of the vector, for example, cultivation of a strain containing the vector in a culture medium containing an antibiotic in case that the selection marker gene is that giving resistance to the antibiotic such as kanamycin. The resultant transformed *Agrobacterium* strain can be cultured in a liquid culture medium, for example, LB medium.

[0081] Soybean, a Chenopodiaceae plant or a Cruciferae plant can be transformed by using thus obtained *Agrobacterium* transformant culture broth as described below. For example, seeds from soybean, beet, rapeseed or mustard are sowed aseptically in, for example, 1/2 MS medium containing 2% sucrose and 0.7% agar. After about 1 week, cotyledons and petioles of the germinated plant are cut off with a scalpel aseptically and transplanted in, for example, MS medium containing 3% sucrose, 0.7% agar, 4.5  $\mu$ M BA, 0.05  $\mu$ M 2,4-D and 3.3  $\mu$ M AgNO<sub>3</sub> and cultured for one day. The cotyledons and petioles thus precultured are transferred to 1000-fold dilution of the above *Agrobacterium* culture broth and allowed to stand for 5 minutes. The cotyledons and petioles are transferred to the same medium as that of the preculture again and cultured for about 3 to 4 days. The cotyledons and petioles thus cultured are transferred to, for example, MS medium containing 3% sucrose, 4.5  $\mu$ M BA, 0.05  $\mu$ M 2,4-D, 3.3  $\mu$ M AgNO<sub>3</sub> and 500 mg/liter cefotaxim, followed by shaking for 1 day to remove microbial cells. The resultant cotyledons and petioles are transferred to, for example, MS medium containing 3% sucrose, 0.7% agar, 4.5  $\mu$ M BA, 0.05  $\mu$ M 2,4-D, 3.3  $\mu$ M AgNO<sub>3</sub> 100 mg/liter cefotaxim and 20 mg/liter kanamycin, followed by culturing for 3 to 4 weeks. Then, the cotyledons and petioles are transferred to, for example, MS medium containing 3% sucrose, 0.7% agar, 4.5  $\mu$ M BA, 0.05  $\mu$ M 2,4-D, 100 mg/liter cefotaxim and 20 mg/liter kanamycin and cultured. Culture in this medium is continued with subculturing every 3 to 4 weeks. When a shoot is regenerated, it is subcultured in, for example, MS medium containing 3% sucrose, 0.7% agar and 20 mg/liter kanamycin for 3 to 4 weeks. When the plant makes roots, it is transferred to vermiculite-peat moss (1 : 1) and acclimatized by culturing at 21 to 22°C under day and night conditions of 12 hours: 12 hours = day time : night. As the plant grows, it is transferred to appropriate cultivation soil to culture the plant. A genomic DNA is extracted from the leaf of the regenerated plant according to the above method and PCR is carried out by using as primers having partial nucleotide sequences of the expression nucleic acid of the present invention to confirm the insertion of the gene of the present invention into the plant.

[0082] As described hereinabove, by introducing the gene of the present invention into a plant, for example, soybean, a Chenopodiaceae plant or a Cruciferae plant, it is possible to vary the expression level and activity of raffinose synthase in the plant to control the amount of raffinose family oligosaccharides in the plant. The gene of the present invention is useful in techniques for varying the expression level and activity of raffinose synthase in soybean, a Chenopodiaceae plant or a Cruciferae plant on the basis of gene homology, for example, techniques such as homologous recombination and antisense technique, cosuppression and the like.

[0083] The following examples further illustrate the present invention in detail but are not to be construed to limit the

scope of the present invention.

#### Exempl 1

##### 5 Preparation of cDNA Derived from Soybean

- [0084] About 2 g of immature seeds of soybean (*Glycine max*) Williams82 were frozen in liquid nitrogen and then ground with a mortar, to which 20 ml of Isogen (Nippon Gene) was added, and the mixture was further thoroughly ground. The ground material was transferred into a centrifugation tube, to which 4 ml of chloroform was added, and the mixture was stirred with a vortex mixer and then centrifuged at 6,500 x g for 10 minutes at 4°C. The water layer was collected, to which 10 ml of isopropanol was added, and the mixture was stirred and then centrifuged at 6,500 x g for 10 minutes at 4°C. The resulting precipitate was washed with 10 ml of 70% ethanol and then dissolved in 1 ml of elution buffer (10 mM Tris-HCl/pH 7.5, 1 mM EDTA, 0.1% SDS). The solution was allowed to stand at 60°C for 10 minutes and then centrifuged at 10,000 x g for 1 minute to remove insoluble matter. To the resulting supernatant was added an equivalent volume of Oligotex-dT30 (Takara), and the mixture was stirred and then allowed to stand at 65°C for 5 minutes. Further, the mixture was placed on ice and allowed to stand for 3 minutes, to which 200 µl of 5 M NaCl was added, and the mixture was mixed and then allowed to stand at 37°C for 10 minutes. The mixture was then centrifuged at 10,000 x g for 3 minutes at 4°C. The precipitate was collected and then suspended in 1 ml of TE buffer, and the suspension was allowed to stand at 65°C for 5 minutes. Further, the suspension was placed on ice and then allowing to stand for 3 minutes, followed by centrifugation at 10,000 x g for 3 minutes at 4°C to remove precipitate.
- [0085] To the resulting supernatant were added 100 µl of 3M sodium acetate and 2 ml of ethanol to precipitate and collect RNA. The collected RNA was washed twice with 70% ethanol and then dissolved in 20 µl of sterilized water, which was used for the subsequent cDNA synthesis. The amount of RNA obtained was determined by the measurement of absorbance at 260 nm.
- [0086] For the cDNA synthesis, First Strand Synthesis Kit for RT-PCR (Amersham) and cDNA Synthesis Kit (Takara) were used, and all operations were made according to the protocol attached to kits.

#### Example 2

##### 30 Cloning of Raffinose Synthase Gene from Soybean cDNA

- [0087] PCR was carried out by using the cDNA obtained from immature seeds of soybean (*Glycine max*) Williams82 in Example 1 as a template and the primers designed on the basis of the amino acid sequence of SEQ ID No: 1, i.e., primers having nucleotide sequences shown in List 4 below to amplify a DNA fragment. The PCR was carried out with Gene Amp PCR Systems 2400 and DNA Thermal Cycler Model 480 of Perkin-Elmer using Advantage KlenTaq cDNA Kit of Clontech. The reaction was carried out by repeating the cycle for maintaining at 94°C for 1 minute, at 50°C for 3 minutes and then at 72°C for 3 minutes 40 times to amplify the DNA fragment. The amplified DNA fragment was cloned with TA cloning kit (Invitrogen), followed by sequence reaction using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit of Perkin-Elmer and analysis of the nucleotide sequence with a 373S DNA sequencer of ABI.
- Based on this sequence, primers having nucleotide sequences shown in List 5 below were synthesized. The synthesis of cDNA was carried out with Marathon Kit of Clontech using mRNA obtained from leaves of soybean Williams82 in Example 1. The cDNA obtained was ligated to an adapter contained in the kit with ligase. These operations were carried out according to the protocol attached to the kit. By using the adapter-ligated cDNA thus prepared, PCR was carried out with the primers shown in List 5 according to the same manner as the above. The nucleotide sequence in terminal region of the gene was analyzed according to the protocol attached to the Marathon Kit of Clontech. As a result, the nucleotide sequence of SEQ ID NO: 2 was determined.

List 4		
4-5-F primer:	cgatggatgg giaaittiat icaiccigai tgggaiaatgt t	41mer
4-6-RV primer:	ggccacatit tiacia(ag)icc iatiggigci aa	32mer
List 5		
5-SC-2:	tgttactagg cgaaacaaga gtatgctctga	30mer

Example 3Preparation of cDNA derived from Chenopodiaceae Plant

- 5 [0088] About 2 g of leaves of beet (*Beta vulgaris*: harning) was frozen in liquid nitrogen and then ground with a mortar, to which 20 ml of Isogen (Nippon Gene) was added, and the mixture was further thoroughly ground. The ground material was transferred into a centrifugation tube, to which 4 ml of chloroform was added, and the mixture was stirred with a vortex mixer and then centrifuged at 6,500 x g for 10 minutes at 4°C. The water layer was collected, to which 10 ml of isopropanol was added, and the mixture was stirred and then centrifuged at 6,500 x g for 10 minutes at 4°C. The resulting precipitate was washed with 10 ml of 70% ethanol and then dissolved in 180 µl of DEPC-treated sterilized water. The solution was allowed to stand at 55°C for 5 minutes and 20 µl of 5 M NaCl was added thereto. The resulting solution was purified using BIOMAG mRNA PURIFICATION KIT (PerSeptive Biosystems: Catalog No. 8-MB4003K).
- 10 [0089] To the resulting mRNA solution were added 3M sodium acetate and ethanol, and RNA was precipitated and collected. The collected RNA was washed twice with 70% ethanol and then dissolved in 20 µl of sterilized water, which was used for the subsequent cDNA synthesis. The amount of RNA obtained was determined by the measurement of absorbance at 260 nm.
- 15 [0090] For the cDNA synthesis, SMART PCR cDNA Synthesis Kit (Clontech) was used, and all operations were made according to the protocol attached to the kit.

20 Example 4Analysis of Nucleotide Sequence of Raffinose Synthase Gene from Chenopodiaceae Plant

- [0091] Synthetic DNA primers having the nucleotide sequences shown in List 6 below were synthesized. The PCR method was carried out with Gene Amp PCR Systems 2400 and DNA Thermal Cycler Model 480 of Perkin-Elmer using Advantage KlenTaq cDNA Kit of Clontech. The PCR was carried out with the above primers and cDNA of beet obtained in the above Example 3 by repeating the cycle for maintaining at 94°C for 1 minute, at 50°C for 3 minutes and then at 72°C for 3 minutes 40 times. As a result, the combinations of primers 6-3-F and 6-8-RV and primers 6-10-F and 6-6-RV gave an amplification of about 0.3 kb and 0.6 kb bands, respectively. The amplified DNA fragments were cloned with TA cloning kit (Invitrogen), followed by sequence reaction using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit of Perkin-Elmer and analysis of nucleotide sequence with a 373S DNA sequencer of ABI. Based on the resulting nucleotide sequences, synthetic DNA primers having nucleotide sequences shown in List 7 below were prepared and PCR was carried out using cDNA obtained from beet in Example 3 in the same manner as above. As a result, DNA having the nucleotide sequence of SEQ ID NO: 4 was finally obtained from cDNA of beet.

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List 6		
6-3-F:	cgaggigggt gccicccigg ittgati atigaigaig gitggca	47mer
6-8-RV:	at(t/c)tt(a/g)tcia cigcia(a/g)(a/g)tc (t/c)tccatigt	29mer
6-10-F:	ggiaicitait gg(c/t)ticaigg itgicaiatg gticaigt	38mer
6-6-RV:	ggccacatit tiacia(a/g)icc iatiggigci aa	32mer
List 7		
7-Sb-1:	atctatttgt catgacgatg atccga	26mer
7-Sb-2RV:	ggccctcatt cccatattgg gatgatcctc	30mer
7-Sb-3RV:	aagcatgccca aacatacaca tgctcaacag	30mer
7-Sb-4RV:	agacccgggg aaagctttgg ggttactact	30mer
7-Sb-5:	tgatgggaa actttatata ccttgact	28mer
7-Sb-6:	gacatgttc catctacaca ccttgtg	28mer
7-Sb-7:	ccaatttatg ttagtgatgt tgtggcaag	30mer
7-Sb-8RV:	tcgactccca gggtagaatt gtcac	26mer

Example 5Preparation of cDNA Derived from Cruciferae Plant

- 5 [0092] About 2 g of leaves of mustard (*Brassica juncea*) was frozen in liquid nitrogen and then ground with a mortar, to which 20 ml of Isogen (Nippon Gene) was added, and the mixture was further thoroughly ground. The ground material was transferred into a centrifugation tube, to which 4ml of chloroform was added, and the mixture was stirred with a vortex mixer and then centrifuged at 6,500 x g for 10 minutes at 4°C. The water layer was collected, to which 10 ml of isopropanol was added, and the mixture was stirred and then centrifuged at 6,500 x g for 10 minutes at 4°C. The resulting precipitate was washed with 10 ml of 70% ethanol and then dissolved in 180 µl of DEPC-treated sterilized water.
- 10 The solution was allowed to stand at 55°C for 5 minutes and to which 20 µl of 5 M NaCl was added. The resulting solution was purified using BIOMAG mRNA PURIFICATION KIT (PerSeptive Biosystems: Catalog No. 8-MB4003K).
- [0093] To the resulting mRNA solution were added 3M sodium acetate and ethanol, and RNA was precipitated and collected. The collected RNA was washed twice with 70% ethanol and then dissolved in 20 µl of sterilized water, which
- 15 was used for the subsequent cDNA synthesis. The amount of RNA obtained was determined by the measurement of absorbance at 260 nm.
- [0094] For the cDNA synthesis, SMART PCR cDNA Synthesis Kit (Clontech) was used, and all operations were carried out according to the protocol attached to the kit.
- [0095] In the same manner as described in the above, mRNA was purified from immature seeds of rapeseed Westar
- 20 (*Brassica napus*) and cDNA was synthesized.

Example 6Isolation and Nucleotide Sequence Analysis of Raffinose Synthase Gene derived from Cruciferae Plant

- 25 [0096] DNA primers having the nucleotide sequences shown in List 8 below were synthesized. PCR was carried out with Gene Amp PCR Systems 2400 and DNA Thermal Cycler Model 480 of Perkin-Elmer using Advantage KlenTaq cDNA Kit of Clontech. The PCR was carried out with the above primers and cDNA of mustard obtained in Example 5 by repeating the cycle for maintaining at 94°C for 1 minute, at 50°C for 3 minutes and then at 72°C for 3 minutes 40 times. The reaction products were analyzed by agarose gel electrophoresis. As a result, an amplification of about the 1.2 kb bands was detected. The amplified DNA fragment was cloned with TA cloning kit (Invitrogen), followed by sequence reaction using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit of Perkin-Elmer and nucleotide sequence analysis with a 373S DNA sequencer of ABI. Based on the resulting nucleotide sequence, synthetic primers having the nucleotide sequences shown in List 9 below were prepared and PCR was carried out using cDNAs
- 30 from mustard (*Brassica juncea*) and rapeseed Westar (*Brassica napus*) obtained in Example 5 according to the same manner as the above. As a result, the nucleotide sequence represented by the 749th to 1215th nucleotides of SEQ ID NO: 6 and by the 1st to 467th nucleotides of SEQ ID NO: 8 were finally determined for cDNA from mustard (*Brassica juncea*) and rapeseed Westar (*Brassica napus*), respectively.
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List 8		
8-#1:	cgattiaaig ttgtgtggac iacicaatgg gtigg	35mer
8-#10RV:	caitgiacca ttgicaccc itgia(ag)ccai taigticc	38mer
List 9		
9-primer-1:	gttaggggttc atatgaacac ctccaagctc	30mer
9-primer-2RV:	caacggcgag atcttgcac gtcaac	26mer

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Example 7Nucleotide Sequence Analysis of Raffinose Synthase Full-Length Gene Derived from Cruciferae Plant

- 55 [0097] Based on the nucleotide sequences obtained in Example 6, DNA primers having the nucleotide sequences shown in List 10 below were synthesized. The cDNAs from mustard (*Brassica juncea*) and rapeseed Westar (*Brassica napus*) obtained in the same manner as described in Example 5 were ligated to adapters contained in Marathon Kit of

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Clontech. By using the adapter-ligated cDNAs thus prepared, PCR was carried out with primers shown in List 10. 10-B-2RV, 10-B-3RV and 10-B-4RV primers were used for nucleotide analysis of 5'-termini, and 10-B-1, 10-B-8, 10-B-7 and 10-B-6 primers were used for nucleotide analysis of 3'-termini. The nucleotide sequences were analyzed according to the protocol attached to the Marathon Kit of Clontech. As a result, the nucleotide sequence of SEQ ID NO: 6 and SEQ ID NO: 8 were determined from mustard (*Brassica juncea*) and rapeseed Westar (*Brassica napus*), respectively.

List 10		
10-B-2RV:	ggattcgaca caaaccgcca cgtcatcgtc	30mer
10-B-3RV:	ccacgtgcac caccogaact tatcgac	27mer
10-B-4RV:	aacatcgata ccatcggagt catgtccaat	30mer
10-B-1:	gttaggggtc atatgaacac cttaagctc	30mer
10-B-8:	tctacgtctg gcacgcgctt tgcggctac	29mer
10-B-7:	gttgacgtca tccacatatt ggagatgtg t	31mer
10-B-6:	gttatcgcta gcatggagca ctgtaatga	29mer

### Example 8

#### Construction of Expression Vectors in Plant for Raffinose Synthase Gene Derived from Cruciferae Plant

[0098] Based on the nucleotide sequence of raffinose synthase gene from mustard obtained in Example 7, DNA primers having the nucleotide sequences shown in List 11 were prepared. By using cDNA of mustard, PCR was carried out in the same manner as described in Example 6. The amplified DNA fragment was digested with *Sac*I. The DNA fragment thus digested was ligated to the vector pBI121(-) previously digested with *Sac*I by using Ligation Kit (Takara). Plasmid pBI121 (Clontech) were digested with *Bam*HI and *Sac*I, and ligated to linkers shown in List 12 to prepare the vector pBI121(+). The vector thus obtained was analyzed by a restriction map and PCR using primers having nucleotide sequences shown in List 13, and confirmed the direction of inserted raffinose synthase gene. The vector whose raffinose synthase gene from mustard was inserted in the expressible direction was designated BjRS-Sac(+)-121 and the one whose raffinose synthase gene from mustard was inserted in the reverse direction was designated BjRS-Sac(-)-121.

List 11		
11-SacI-BjN:	aacgagctca atccaaaatc tcatcaaata atcgc	35mer
11-SacI-BjintRV:	acaatagttg agggcggaag agtag	25mer
List 12		
12-BamSac-(+)linker:	gatcgagctc gtgtcggatc cagct	25mer
12-BamSac-(-)linker:	ggatccgaca cgagctc	17mer
List 13		
13-35S-3:	cctcctcgga ttccattgcc cagctatctg	30mer
13-B-2RV:	ggattcgaca caaaccgcca cgtcatcgtc	30mer
13-B-8:	tctacgtctg gcacgcgctt tgcggctac	29mer

## Exempl 9

## Transformation with Raffinose Synthase Gen D rived from Crucifera Plant



[0099] The vectors BjRS-Sac(+)-121 and BjRS-Sac(-)-121 prepared in Example 8 were used for the transformation of mustard (*Brassica juncea*) by the *Agrobacterium* infection method.

[0100] *Agrobacterium tumefaciens* (strain LBA4404 having rifampicin and streptomycin resistance) previously converted into a competent state by calcium chloride treatment was transformed independently with two plasmids BjRS-Sac (+) -121 and BjRS-Sac(-)-121 prepared in Example 8. The transformants were selected on LB medium containing 50 µg/ml rifampicin and 25 µg/ml kanamycin by utilizing the kanamycin resistant character conferred by the kanamycin resistant gene (neomycin phosphotransferase, NPTII) of the introduced plasmids.

[0101] The transformant *Agrobacterium* obtained (*Agrobacterium tumefaciens* strain LBA4404; rifampicin and streptomycin resistant) was cultured on LB medium containing 50 µg/ml rifampicin and 25 µg/ml kanamycin at 28°C for a whole day and night, and the culture was used for the transformation of mustard by the method described below.

[0102] The seeds of mustard were aseptically sowed on 1/2 MS medium containing 2% sucrose and 0.7% agar. After one week, cotyledons and petioles of sprouting plants were cut out with a scalpel, and transferred to MS medium containing 3% sucrose, 0.7% agar, 4.5 µM BA, 0.05 µM 2,4-D and 3.3 µM AgNO<sub>3</sub>, followed by preculture for 1 day. The precultured cotyledons and petioles were transferred in a 1000-fold dilution of the *Agrobacterium* culture broth and allowed to stand for 5 minutes. The cotyledons and petioles were transferred again to the same medium as used in the preculture, and cultured for 3 to 4 days. The cultured cotyledons and petioles were transferred to MS medium containing 3% sucrose, 4.5 µM BA, 0.05 µM 2,4-D, 3.3 µM AgNO<sub>3</sub> and 500 mg/l cefotaxim, and shaken for 1 day to remove microbial cells. The cotyledons and petioles thus treated were transferred to MS medium containing 3% sucrose, 0.7% agar, 4.5 µM BA, 0.05 µM 2,4-D, 3.3 µM AgNO<sub>3</sub>, 100 mg/l cefotaxim and 20 mg/l kanamycin, and cultured for 3 to 4 weeks. The cotyledons and petioles were transferred to MS medium containing 3% sucrose, 0.7% agar, 4.5 µM BA, 0.05 µM 2,4-D, 100 mg/l cefotaxim and 20 mg/l kanamycin, and cultivated. The cultivation on this medium was continued with subculturing at intervals of 3 to 4 weeks. When shoots are began to regenerate, these shoots are subcultured on MS medium containing 3% sucrose, 0.7% agar and 20 mg/l kanamycin, and cultivated for 3 to 4 weeks. The rooting plants are transferred to vermiculite : peat moss = 1 : 1, and cultivated at 21°C to 22°C in a cycle of day/night = 12 hours : 12 hours. With the progress of plant body growth, the plants are grown with cultivation soil.

## BRIEF DESCRIPTION OF SEQUENCES

## [0103]

SEQ ID NO: 1 shows an amino acid sequence of a raffinose synthase protein encoded by the raffinose synthase gene of the present invention.

SEQ ID NO: 2 shows a nucleotide sequence of the raffinose synthase gene of the present invention.

SEQ ID NO: 3 shows an amino acid sequence of a raffinose synthase protein encoded by the raffinose synthase gene obtained from beet.

SEQ ID NO: 4 shows a cDNA nucleotide sequence of the raffinose synthase gene obtained from beet.

SEQ ID NO: 5 shows an amino acid sequence of a raffinose synthase protein encoded by the raffinose synthase gene obtained from mustard.

SEQ ID NO: 6 shows a cDNA nucleotide sequence of the raffinose synthase gene obtained from mustard.

SEQ ID NO: 7 shows an amino acid sequence of a raffinose synthase protein encoded by the raffinose synthase gene obtained from rapeseed.

SEQ ID NO: 8 shows a cDNA nucleotide sequence of the raffinose synthase gene obtained from rapeseed.

## List 1:

[0104] The nucleotide sequences shown in List 1 are examples of the typical primers used in the amplification of a DNA fragment having a raffinose synthase gene. All of these sequences are based on the nucleotide sequence of SEQ ID NO: 2. Primer 11 is a sense primer and Primer 12 is an antisense primer. Depending upon the purpose, recognition sequences for suitable restriction enzymes can be added to the 5'-termini of these nucleotide sequences.

## List 2:

[0105] The nucleotide sequences shown in List 2 are examples of the typical primers used in the amplification of a cDNA of a raffinose synthase gene. Primer 21 is a sense primer corresponding to the 5'-terminus of the beet-derived



raffinose synthase gene. Primer 22 is an antisense primer corresponding to the 3'-terminus. Depending upon the purpose, recognition sequences for suitable restriction enzymes can be added to the 5'-termini of these nucleotide sequences.

5 [0106] Primer 23 is a sense primer corresponding to the N-terminus of the open reading frame, and primer 24 is an antisense primer corresponding to the C-terminus.

List 3:

10 [0107] Among the nucleotide sequences shown in List 3, primers 31 and 32 are typical primers used in the amplification of a DNA having the partial nucleotide sequence of a raffinose synthase gene. Primer 31 is a sense primer used in the amplification of a DNA having the partial nucleotide sequence of a raffinose synthase gene from mustard and rapeseed and primer 32 is an antisense primer. Depending upon the purpose, recognition sequences for suitable restriction enzymes can be added to the 5'-termini of these nucleotide sequences.

15 [0108] Primers 33 and 34 are the typical primers used in the amplification of a cDNA of a raffinose synthase gene of mustard. Primers 33 and 34 are both based on the nucleotide sequence of raffinose synthase gene in the non-translated region. Primer 33 is a sense primer corresponding to the 5'-terminal non-translated region of the mustard-derived raffinose synthase gene. Primer 34 is an antisense primer corresponding to the 3'-terminal non-translated region.

20 [0109] Primers 35 and 36 are typical primers used in the amplification of an open reading frame coding for the amino acid sequence of a raffinose synthase protein in the cDNA of a raffinose synthase gene. Primer 35 is a sense primer corresponding to the 5'-terminus of the above open reading frame. Primer 36 is an antisense primer corresponding to the 3'-terminus. Depending upon the purpose, recognition sequences for suitable restriction enzymes can be added to the 5'-termini of these nucleotide sequences.

List 4:

25 [0110] The nucleotide sequences shown in List 4 are of the primers used in the cloning of a DNA fragment having the present raffinose synthase gene. The base represented by the symbol "i" is inosine. The bases shown in parentheses mean that a mixture of those bases is used in the synthesis. The symbol "RV" as used after the primer number means that the primer referred to by this symbol has an antisense sequence.

List 5:

30 [0111] The nucleotide sequence shown in List 5 is of the primer used in the analysis of a nucleotide sequence of the present raffinose synthase gene. 5-SC-2 is used in the analysis of the present nucleotide sequence in the 3'-terminal region.

List 6:

40 [0112] The nucleotide sequences shown in List 6 are of the primers used in the analysis of the present raffinose synthase gene of beet. The base represented by the symbol "i" is inosine. The bases shown in parentheses mean that a mixture of those bases was used in the synthesis. The symbol "RV" as used after the primer number means that the primer referred to by this symbol has an antisense sequence.

List 7:

45 [0113] The nucleotide sequences shown in List 7 are of the primers synthesized on the partial nucleotide sequences of the beet raffinose synthase gene. The symbol "RV" as used after the primer number means that the primer referred to by this symbol has an antisense sequence.

50 List 8:

55 [0114] The nucleotide sequences shown in List 8 are of the primers used in the analysis of the cDNA nucleotide sequence of a raffinose synthase gene of mustard. The base represented by the symbol "i" is inosine. The bases shown in parentheses mean that a mixture of those bases. The symbol "RV" as used after the primer number means that the primer referred to by this symbol has an antisense sequence.

List 9:

[0115] The nucleotide sequences shown in List 9 are of the primers synthesized on the partial nucleotide sequences of the mustard raffinose synthase gene. The symbol "RV" as used after the primer number means that the primer referred to by this symbol has an antisense sequence.

List 10:

[0116] The nucleotide sequences shown in List 10 are of the primers used in the analysis of the nucleotide sequences of raffinose synthase gene of mustard and rapeseed. The symbol "RV" as used after the primer number means that the primer referred to by this symbol has an antisense sequence.

List 11:

[0117] The nucleotide sequences shown in List 11 are of the primers used in the amplification of 5-terminal region of a mustard raffinose synthase gene. 11-SacI-BjN is a primer whose SacI restriction site is added to the nucleotide sequence represented by the 4th to 29th nucleotides in SEQ ID NO: 6. 11-SacI-BjintRV is an antisense primer having a nucleotide sequence corresponding to the nucleotide sequence represented by the 1164th to 1188th nucleotides in SEQ ID NO: 6.

List 12:

[0118] The nucleotide sequences shown in List 12 are of the adapters added to a mustard cDNA. These synthetic DNA take a double-stranded form when mixed together because they are complementary strands. This adapter has cohesive ends of cleavage sites for the restriction enzymes BamHI and SacI on both termini, and contains the restriction sites for the restriction enzymes BamHI and SacI in the double-stranded region.

List 13:

[0119] The nucleotide sequences shown in List 13 are of the primers used in the confirmation of inserting direction of the mustard-derived raffinose synthase gene. 13-35S-3 is a primer of sense to 35S promoter. 13-B-2RV is an antisense primer having the nucleotide sequence represented by the 593rd to 622nd nucleotides of SEQ ID NO: 6, 13-B-8 is a sense primer having the nucleotide sequence represented by the 1110th to 1138th nucleotides in SEQ ID NO: 6.

[0120] As described hereinabove, according to the present invention, it is possible to provide raffinose synthase genes which can be utilized in techniques for varying expression level and activity of raffinose synthase in plants.

#### SEQUENCE LISTING FREE TEXT

[0121] SEQ ID NO: 9 to SEQ ID NO: 20: Designed oligonucleotide primer to obtain raffinose synthase gene.

[0122] SEQ ID NO: 21 and SEQ ID NO: 22: Designed oligonucleotide primer to obtain raffinose synthase gene, n is i, r is a or g.

[0123] SEQ ID NO: 23: Designated oligonucleotide primer to obtain raffinose synthase gene.

[0124] SEQ ID NO: 24 to SEQ ID NO: 27: Designed oligonucleotide primer to obtain raffinose synthase gene, n is i, y is t or c, r is a or g.

[0125] SEQ ID NO: 28 to SEQ ID NO: 35: Designed oligonucleotide primer to obtain raffinose synthase gene.

[0126] SEQ ID NO: 36 and SEQ ID NO: 37: Designed oligonucleotide primer to obtain raffinose synthase gene, n is i, r is a or g.

[0127] SEQ ID NO: 38 to SEQ ID NO: 48: Designed oligonucleotide primer to obtain raffinose synthase gene.

[0128] SEQ ID NO: 49 and SEQ ID NO: 50: Designed oligonucleotide linker to obtain raffinose synthase gene.

[0129] SEQ ID NO: 51 to SEQ ID NO: 53: Designed oligonucleotide primer to confirm direction of the inserted raffinose synthase gene.

SEQUENCE LISTING

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<110> Eijiro WATANABE et al.; Sumitomo Chemical Company Limited

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35

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45

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55

60

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75

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	70 75 80	
40	aag gga att ccc ttc atg agt atc ttc agg ttc aag gta tgg tgg act	526
	Lys Gly Ile Pro Phe Met Ser Ile Phe Arg Phe Lys Val Trp Trp Thr	
	85 90 95	
	acc cat tgg act ggg tcc aat ggg cgg gac ctt gag cat gag acc caa	574
45	Thr His Trp Thr Gly Ser Asn Gly Arg Asp Leu Glu His Glu Thr Gln	
	100 105 110	
	att ctc atc ctt gat aag tca gat gaa ggt ttg ggc cgt ccc tat att	622
	Ile Leu Ile Leu Asp Lys Ser Asp Glu Gly Leu Gly Arg Pro Tyr Ile	
	115 120 125	
50	gtg atc ctc cca ttg atc gaa ggc cca ttt cgg gca tct ctc cag ccg	670
	Val Ile Leu Pro Leu Ile Glu Gly Pro Phe Arg Ala Ser Leu Gln Pro	

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	130	135	140	145	
	ggt tct gtt gat gac tat gtg gat ata tgt gtt gag agt ggg tcc act				718
5	Gly Ser Val Asp Asp Tyr Val Asp Ile Cys Val Glu Ser Gly Ser Thr				
	150	155	160		
	aaa gtt gtc gga gac tcg ttc cgg gct gtt ctt tat ata cgg gct ggg				766
10	Lys Val Val Gly Asp Ser Phe Arg Ala Val Leu Tyr Ile Arg Ala Gly				
	165	170	175		
	cct gac cca ttt aag tta att aaa gat aca atg aag gaa gtc caa gcc				814
	Pro Asp Pro Phe Lys Leu Ile Lys Asp Thr Met Lys Glu Val Gln Ala				
	180	185	190		
15	cat tta ggg act ttc aaa ctc tta gat gac aaa act cct cca gga ata				862
	His Leu Gly Thr Phe Lys Leu Leu Asp Asp Lys Thr Pro Pro Gly Ile				
	195	200	205		
20	gtg gac aag ttt gga tgg tgt aca tgg gat gca ttt tac ctc aaa gta				910
	Val Asp Lys Phe Gly Trp Cys Thr Trp Asp Ala Phe Tyr Leu Lys Val				
	210	215	220	225	
	gag ccw tat ggt gtt tgg gaa gga gtt aaa gga ctc gtc gaa aac ggg				958
25	Glu Pro Tyr Gly Val Trp Glu Gly Val Lys Gly Leu Val Glu Asn Gly				
	230	235	240		
	gtc cca ccc ggt ctc gta ctc att gat gat ggg tgg caa tct att tgt				1006
	Val Pro Pro Gly Leu Val Leu Ile Asp Asp Gly Trp Gln Ser Ile Cys				
30	245	250	255		
	cat gac gat gat ccg att acc gac caa gaa ggg ata aac cgg act tct				1054
	His Asp Asp Asp Pro Ile Thr Asp Gln Glu Gly Ile Asn Arg Thr Ser				
	260	265	270		
35	gcc ggc gag caa atg cca tgt aga ttg atc aag tac gag gaa aac ttc				1102
	Ala Gly Glu Gln Met Pro Cys Arg Leu Ile Lys Tyr Glu Glu Asn Phe				
	275	280	285		
40	aag ttt agg gac tat aaa agc cca aat att atg ggc cat gag gat cat				1150
	Lys Phe Arg Asp Tyr Lys Ser Pro Asn Ile Met Gly His Glu Asp His				
	290	295	300	305	
	ccc aat atg gga atg agg gcc ttt gtt agg gac ctt aag gag gag ttc				1198
45	Pro Asn Met Gly Met Arg Ala Phe Val Arg Asp Leu Lys Glu Glu Phe				
	310	315	320		
	aaa act gtt gag cat gtg tat gtt tgg cat gct ttt acg ggc tat tgg				1246
	Lys Thr Val Glu His Val Tyr Val Trp His Ala Phe Thr Gly Tyr Trp				
50	325	330	335		
	gga ggg gta agg ccc aat gtt cca ggc cta ccr gag gcc caa gta gta				1294
	Gly Gly Val Arg Pro Asn Val Pro Gly Leu Pro Glu Ala Gln Val Val				

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	340	345	350	
	acc cca aag ctt tcc ccg ggt ctt gag atg aca atg gaa gat cta gct			1342
5	Thr Pro Lys Leu Ser Pro Gly Leu Glu Met Thr Met Glu Asp Leu Ala			
	355	360	365	
	gtg gat aaa att gtt aat aat ggt att ggg ctt gtc cag cct gat aag			1390
	Val Asp Lys Ile Val Asn Asn Gly Ile Gly Leu Val Gln Pro Asp Lys			
10	370	375	380	385
	gcc caa gaa ctt tat gaa ggg ttg cat tct cat ttg gaa aat tgt ggg			1438
	Ala Gln Glu Leu Tyr Glu Gly Leu His Ser His Leu Glu Asn Cys Gly			
	390	395	400	
15	att gat gga gtc aaa gtt gat gtc atc cat ttg ttg gag atg atg gca			1486
	Ile Asp Gly Val Lys Val Asp Val Ile His Leu Leu Glu Met Met Ala			
	405	410	415	
20	gag gac tat gga gga aga gtt gaa cta gca aaa aca tac tat aag gca			1534
	Glu Asp Tyr Gly Gly Arg Val Glu Leu Ala Lys Thr Tyr Tyr Lys Ala			
	420	425	430	
	ata aca gaa tca gtg cgt aag cat ttc aaa ggc aac ggt gtg att gct			1582
25	Ile Thr Glu Ser Val Arg Lys His Phe Lys Gly Asn Gly Val Ile Ala			
	435	440	445	
	agc atg gag cag tgc aac gat ttc atg ctc ctt ggt act gag acc att			1630
	Ser Met Glu Gln Cys Asn Asp Phe Met Leu Leu Gly Thr Glu Thr Ile			
30	450	455	460	465
	tgt ctt ggt cgc gtt ggg gat gac ttt tgg cca act gat ccg tct gga			1678
	Cys Leu Gly Arg Val Gly Asp Asp Phe Trp Pro Thr Asp Pro Ser Gly			
	470	475	480	
35	gat ata aat ggt aca tat tgg ctc caa ggc tgt cat atg gtg cat tgt			1726
	Asp Ile Asn Gly Thr Tyr Trp Leu Gln Gly Cys His Met Val His Cys			
	485	490	495	
40	gcc tac aat agc tta tgg atg gga aac ttt ata cac cct gac tgg gac			1774
	Ala Tyr Asn Ser Leu Trp Met Gly Asn Phe Ile His Pro Asp Trp Asp			
	500	505	510	
	atg ttc caa tct aca cac cct tgt gct gaa ttt cat gct gca tct cgt			1822
45	Met Phe Gln Ser Thr His Pro Cys Ala Glu Phe His Ala Ala Ser Arg			
	515	520	525	
	gcg att tct ggt gga cca att tat gtt agt gat gtt gtt ggc aag cat			1870
	Ala Ile Ser Gly Gly Pro Ile Tyr Val Ser Asp Val Val Gly Lys His			
50	530	535	540	545
	aac atc ccc ttg ctc aaa agg ctc gtc ttg gct gat ggt tcg atc ctt			1918
	Asn Ile Pro Leu Leu Lys Arg Leu Val Leu Ala Asp Gly Ser Ile Leu			

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	550	555	560	
	cgt tgc gag tac cat gca ctt cct act aag gat tgc cta ttt gta gat			1966
5	Arg Cys Glu Tyr His Ala Leu Pro Thr Lys Asp Cys Leu Phe Val Asp			
	565	570	575	
	cct ttg cac gat ggc aaa aca atg ctc aaa att tgg aac ctc aac aag			2014
10	Pro Leu His Asp Gly Lys Thr Met Leu Lys Ile Trp Asn Leu Asn Lys			
	580	585	590	
	tac aat gga gtg ctt gga gtc ttc aat tgc caa gga gga ggg tgg agc			2062
	Tyr Asn Gly Val Leu Gly Val Phe Asn Cys Gln Gly Gly Gly Trp Ser			
	595	600	605	
15	cgt gag tct cga aaa aat cta tgt ttc tca gag tat tca aaa cct att			2110
	Arg Glu Ser Arg Lys Asn Leu Cys Phe Ser Glu Tyr Ser Lys Pro Ile			
	610	615	620	625
20	tcc tgc aag aca agt cca aaa gat gtt gaa tgg gag aac gga cac aag			2158
	Ser Cys Lys Thr Ser Pro Lys Asp Val Glu Trp Glu Asn Gly His Lys			
	630	635	640	
25	cca ttc ccc atc aaa gga gtg gaa tgt ttt gcc atg tac ttc acc aag			2206
	Pro Phe Pro Ile Lys Gly Val Glu Cys Phe Ala Met Tyr Phe Thr Lys			
	645	650	655	
	gaa aaa aag cta atc ctc tca caa cta tct gac acc att gaa ata tca			2254
	Glu Lys Lys Leu Ile Leu Ser Gln Leu Ser Asp Thr Ile Glu Ile Ser			
30	660	665	670	
	ctt gat ccc ttc gat tac gag ctt att gta gtc tct ccg atg aca att			2302
	Leu Asp Pro Phe Asp Tyr Glu Leu Ile Val Val Ser Pro Met Thr Ile			
	675	680	685	
35	cta ccc tgg gag tcg atc gca ttt gca ccc ata gga tta gta aac atg			2350
	Leu Pro Trp Glu Ser Ile Ala Phe Ala Pro Ile Gly Leu Val Asn Met			
	690	695	700	705
40	ctc aac gcc gga ggg gca gtc aag tct ttg gac atc agt gag gat aat			2398
	Leu Asn Ala Gly Gly Ala Val Lys Ser Leu Asp Ile Ser Glu Asp Asn			
	710	715	720	
	gag gat aag atg gtt cag gtt ggt att aaa ggg gcc gga gaa atg atg			2446
45	Glu Asp Lys Met Val Gln Val Gly Ile Lys Gly Ala Gly Glu Met Met			
	725	730	735	
	gtt tat tca tca gaa aag cca aaa gcg tgt aga gtt aat gga gaa gac			2494
	Val Tyr Ser Ser Glu Lys Pro Lys Ala Cys Arg Val Asn Gly Glu Asp			
50	740	745	750	
	atg gag ttt gag tat gaa gag agc atg att aag gtt caa gtt aca tgg			2542
	Met Glu Phe Glu Tyr Glu Glu Ser Met Ile Lys Val Gln Val Thr Trp			

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	755	760	765	
	aac cat aac tca ggt ggt ttt acc act gtt gag tac tta ttt tga gcttg			2592
5	Asn His Asn Ser Gly Gly Phe Thr Thr Val Glu Tyr Leu Phe			
	770	775	780	
	aagctaattct aagtcctttac ttaatgagtg atgtaaactga gtagttgact tgagagtaca			2652
	gtatgtgtga agcttattat tccaaaaaaaa aaaaaaaaaa			2690

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	Asp	Leu	Ser	Gly	Lys	Pro	Leu	Phe	Arg	Leu	Glu	Gly	Ser	Asp	Leu	Leu
				20					25					30		
25	Ala	Asn	Gly	His	Val	Val	Leu	Thr	Asp	Val	Pro	Val	Asn	Val	Thr	Val
			35					40						45		
	Thr	Ala	Ser	Pro	Tyr	Leu	Ala	Asp	Lys	Asp	Gly	Glu	Pro	Val	Asp	Ala
		50					55					60				
30	Ser	Ala	Gly	Ser	Phe	Ile	Gly	Phe	Asn	Leu	Asp	Gly	Glu	Pro	Arg	Ser
	65					70					75					80
	Arg	His	Val	Ala	Ser	Ile	Gly	Lys	Leu	Arg	Asp	Ile	Arg	Phe	Met	Ser
					85					90					95	
35	Ile	Phe	Arg	Phe	Lys	Val	Trp	Trp	Thr	Thr	His	Trp	Val	Gly	Ser	Lys
				100					105					110		
	Gly	Ser	Asp	Ile	Glu	Asn	Glu	Thr	Gln	Ile	Ile	Ile	Leu	Glu	Asn	Ser
		115						120					125			
40	Gly	Ser	Gly	Arg	Pro	Tyr	Val	Leu	Leu	Leu	Pro	Leu	Leu	Glu	Gly	Ser
		130					135					140				
	Phe	Arg	Ser	Ser	Phe	Gln	Pro	Gly	Glu	Asp	Asp	Asp	Val	Ala	Val	Cys
45	145					150					155					160
	Val	Glu	Ser	Gly	Ser	Thr	Gln	Val	Thr	Gly	Ser	Glu	Phe	Arg	Gln	Val
				165						170					175	
	Val	Tyr	Val	His	Ala	Gly	Asp	Asp	Pro	Phe	Lys	Leu	Val	Lys	Asp	Ala
50				180					185					190		
	Met	Lys	Val	Val	Arg	Val	His	Met	Asn	Thr	Phe	Lys	Leu	Leu	Glu	Glu
				195				200					205			

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Lys Thr Pro Pro Gly Ile Val Asp Lys Phe Gly Trp Cys Thr Trp Asp  
 210 215 220  
 5 Ala Phe Tyr Leu Thr Val Asn Pro Asp Gly Val His Lys Gly Val Lys  
 225 230 235 240  
 Cys Leu Val Asp Gly Gly Cys Pro Pro Gly Leu Val Leu Ile Asp Asp  
 245 250 255  
 10 Gly Trp Gln Ser Ile Gly His Asp Ser Asp Gly Ile Asp Val Glu Gly  
 260 265 270  
 Met Ser Cys Thr Val Ala Gly Glu Gln Met Pro Cys Arg Leu Leu Lys  
 275 280 285  
 15 Phe Gln Glu Asn Phe Lys Phe Arg Asp Tyr Val Ser Pro Lys Asp Lys  
 290 295 300  
 Asn Glu Val Gly Met Lys Ala Phe Val Arg Asp Leu Lys Glu Glu Phe  
 305 310 315 320  
 20 Ser Thr Val Asp Tyr Ile Tyr Val Trp His Ala Leu Cys Gly Tyr Trp  
 325 330 335  
 Gly Gly Leu Arg Pro Gly Ala Pro Thr Leu Pro Pro Ser Thr Ile Val  
 340 345 350  
 25 Arg Pro Glu Leu Ser Pro Gly Leu Lys Leu Thr Met Gln Asp Leu Ala  
 355 360 365  
 Val Asp Lys Ile Val Asp Thr Gly Ile Gly Phe Val Ser Pro Asp Met  
 370 375 380  
 30 Ala Asn Glu Phe Tyr Glu Gly Leu His Ser His Leu Gln Asn Val Gly  
 385 390 395 400  
 Ile Asp Gly Val Lys Val Asp Val Ile His Ile Leu Glu Met Leu Cys  
 405 410 415  
 35 Glu Lys Tyr Gly Gly Arg Val Asp Leu Ala Lys Ala Tyr Phe Lys Ala  
 420 425 430  
 Leu Thr Ser Ser Val Asn Lys His Phe Asp Gly Asn Gly Val Ile Ala  
 435 440 445  
 40 Ser Met Glu His Cys Asn Asp Phe Met Phe Leu Gly Thr Glu Ala Ile  
 450 455 460  
 Ser Leu Gly Arg Val Gly Asp Asp Phe Trp Cys Thr Asp Pro Ser Gly  
 465 470 475 480  
 45 Asp Ile Asn Gly Thr Tyr Trp Leu Gln Gly Cys His Met Val His Cys  
 485 490 495  
 50 Ala Tyr Asn Ser Leu Trp Met Gly Asn Phe Ile Gln Pro Asp Trp Asp  
 500 505 510  
 Met Phe Gln Ser Thr His Pro Cys Ala Glu Phe His Ala Ala Ser Arg

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515                      520                      525  
 5    Ala Ile Ser Gly Gly Pro Ile Tyr Ile Ser Asp Cys Val Gly Gln His  
      530                      535                      540  
   Asp Phe Asp Leu Leu Lys Arg Leu Val Leu Pro Asp Gly Ser Ile Leu  
 545                      550                      555                      560  
 10   Arg Cys Glu His Tyr Ala Leu Pro Thr Arg Asp Arg Leu Phe Glu Asp  
      565                      570                      575  
   Pro Leu His Asp Gly Lys Thr Met Leu Lys Ile Trp Asn Leu Asn Lys  
      580                      585                      590  
 15   Tyr Thr Gly Ile Ile Gly Ala Phe Asn Cys Gln Gly Gly Gly Trp Cys  
      595                      600                      605  
   Arg Glu Thr Arg Arg Asn Gln Cys Phe Ser Gln Cys Val Asn Thr Leu  
      610                      615                      620  
 20   Thr Ala Thr Thr Asn Pro Lys Asp Val Glu Trp Asn Ser Gly Asn Asn  
      625                      630                      635                      640  
   Pro Ile Ser Val Glu Asn Val Glu Glu Phe Ala Leu Phe Leu Ser Gln  
      645                      650                      655  
 25   Ser Lys Lys Leu Val Leu Ser Gly Pro Asn Asp Asp Leu Glu Ile Thr  
      660                      665                      670  
   Leu Glu Pro Phe Lys Phe Glu Leu Ile Thr Val Ser Pro Val Val Thr  
      675                      680                      685  
 30   Ile Glu Gly Ser Ser Val Gln Phe Ala Pro Ile Gly Leu Val Asn Met  
      690                      695                      700  
   Leu Asn Thr Ser Gly Ala Ile Arg Ser Leu Val Tyr His Glu Glu Ser  
 35   705                      710                      715                      720  
   Val Glu Ile Gly Val Arg Gly Ala Gly Glu Phe Arg Val Tyr Ala Ser  
      725                      730                      735  
 40   Arg Lys Pro Ala Ser Cys Lys Ile Asp Gly Glu Val Val Glu Phe Gly  
      740                      745                      750  
   Tyr Glu Glu Ser Met Val Met Val Gln Val Pro Trp Ser Ala Pro Glu  
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 45   Gly Leu Ser Ser Ile Lys Tyr Glu Phe  
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	tctatcagcg aaa atg gct cca ccg agc gta att aaa tcc gat gct gca	169
	Met Ala Pro Pro Ser Val Ile Lys Ser Asp Ala Ala	
	5 10	
15	gtc aac ggc att gac ctc tcc gga aag ccg ctt ttc cgg cta gag ggt	217
	Val Asn Gly Ile Asp Leu Ser Gly Lys Pro Leu Phe Arg Leu Glu Gly	
	15 20 25	
20	tcc gat ctc cta gcc aat ggt cac gtt gtc tta acc gat gta ccg gtt	265
	Ser Asp Leu Leu Ala Asn Gly His Val Val Leu Thr Asp Val Pro Val	
	30 35 40	
25	aac gtg act gtc act gct tca cct tac cta gct gac aaa gac gga gaa	313
	Asn Val Thr Val Thr Ala Ser Pro Tyr Leu Ala Asp Lys Asp Gly Glu	
	45 50 55 60	
	ccg gtt gac gcc tcc gct ggt tca ttc atc ggg ttt aat ctc gac ggt	361
	Pro Val Asp Ala Ser Ala Gly Ser Phe Ile Gly Phe Asn Leu Asp Gly	
	65 70 75	
30	gag cca cga agc cgc cac gtg gcg tcc atc ggt aaa ctc agg gat att	409
	Glu Pro Arg Ser Arg His Val Ala Ser Ile Gly Lys Leu Arg Asp Ile	
	80 85 90	
35	cga ttc atg agc ata ttc cgt ttc aag gtt tgg tgg act act cac tgg	457
	Arg Phe Met Ser Ile Phe Arg Phe Lys Val Trp Trp Thr Thr His Trp	
	95 100 105	
40	gtc ggt tcc aaa gga tcc gac atc gag aac gag acc cag atc atc atc	505
	Val Gly Ser Lys Gly Ser Asp Ile Glu Asn Glu Thr Gln Ile Ile Ile	
	110 115 120	
	ctc gag aac tcc ggg tcg ggt cgt cct tat gtt ctt ctt ctg ccg ctt	553
	Leu Glu Asn Ser Gly Ser Gly Arg Pro Tyr Val Leu Leu Leu Pro Leu	
45	125 130 135 140	
	ctt gaa ggc tct ttc cgt tca tcc ttt cag cct ggg gaa gac gat gac	601
	Leu Glu Gly Ser Phe Arg Ser Ser Phe Gln Pro Gly Glu Asp Asp Asp	
	145 150 155	
50	gtg gcg gtt tgt gtc gaa tcc ggg tcg acc cag gtg acc ggg tcg gag	649
	Val Ala Val Cys Val Glu Ser Gly Ser Thr Gln Val Thr Gly Ser Glu	

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	160	165	170	
	ttt cgt caa gtt gtg tat gtt cac gcc gga gac gat ccg ttc aag ctc			697
5	Phe Arg Gln Val Val Tyr Val His Ala Gly Asp Asp Pro Phe Lys Leu			
	175	180	185	
	gtg aaa gac gcg atg aag gtg gtt agg gtt cat atg aac acc ttc aag			745
	Val Lys Asp Ala Met Lys Val Val Arg Val His Met Asn Thr Phe Lys			
10	190	195	200	
	ctc ttg gaa gag aag acr ccg ccg gga atc gtc gat aag ttc ggg tgg			793
	Leu Leu Glu Glu Lys Thr Pro Pro Gly Ile Val Asp Lys Phe Gly Trp			
	205	210	215	220
15	tgc acg tgg gat gcg ttt tat ttg acg gtg aac cct gac gga gtt cat			841
	Cys Thr Trp Asp Ala Phe Tyr Leu Thr Val Asn Pro Asp Gly Val His			
	225	230	235	
20	aag ggt gtt aag tgt ctc gtc gac ggt ggt tgt ccg ccg gga ttg gtc			889
	Lys Gly Val Lys Cys Leu Val Asp Gly Gly Cys Pro Pro Gly Leu Val			
	240	245	250	
	cta atc gac gac ggt tgg caa tcg att gga cat gac tcc gat ggt atc			937
25	Leu Ile Asp Asp Gly Trp Gln Ser Ile Gly His Asp Ser Asp Gly Ile			
	255	260	265	
	gat gtt gaa ggg atg agt tgt acc gtc gcc ggg gag caa atg cct tgc			985
	Asp Val Glu Gly Met Ser Cys Thr Val Ala Gly Glu Gln Met Pro Cys			
30	270	275	280	
	agg ctt ctg aaa ttt caa gag aac ttc aag ttc aga gac tac gtc tct			1033
	Arg Leu Leu Lys Phe Gln Glu Asn Phe Lys Phe Arg Asp Tyr Val Ser			
	285	290	295	300
35	ccg aaa gac aaa aac gaa gtc ggg atg aaa gct ttc gtc aga gat ctg			1081
	Pro Lys Asp Lys Asn Glu Val Gly Met Lys Ala Phe Val Arg Asp Leu			
	305	310	315	
	aaa gaa gaa ttc tcc acc gtt gat tac atc tac gtc tgg cac gcg ctt			1129
40	Lys Glu Glu Phe Ser Thr Val Asp Tyr Ile Tyr Val Trp His Ala Leu			
	320	325	330	
	tgc ggc tac tgg ggt ggt ctt cgt ccc gga gct cct act ctt ccg ccc			1177
45	Cys Gly Tyr Trp Gly Gly Leu Arg Pro Gly Ala Pro Thr Leu Pro Pro			
	335	340	345	
	tca act att gtc cgg cca gag ctc tcg ccg ggg ctt aag ttg acg atg			1225
	Ser Thr Ile Val Arg Pro Glu Leu Ser Pro Gly Leu Lys Leu Thr Met			
	350	355	360	
50	caa gat ctc gcc gtt gat aag att gtc gat acc gga atc gga ttc gtc			1273
	Gln Asp Leu Ala Val Asp Lys Ile Val Asp Thr Gly Ile Gly Phe Val			

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	365	370	375	380	
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5	Ser Pro Asp Met Ala Asn Glu Phe Tyr Glu Gly Leu His Ser His Leu				
		385	390	395	
	caa aac gtc ggt att gac ggc gtt aaa gtt gac gtc atc cac ata ttg				1369
10	Gln Asn Val Gly Ile Asp Gly Val Lys Val Asp Val Ile His Ile Leu				
		400	405	410	
	gag atg ttg tgc gag aaa tat ggc ggg aga gta gac ttg gct aaa gct				1417
	Glu Met Leu Cys Glu Lys Tyr Gly Gly Arg Val Asp Leu Ala Lys Ala				
		415	420	425	
15	tac ttc aag gcg tta act tcc tca gtg aat aag cat ttt gac ggt aac				1465
	Tyr Phe Lys Ala Leu Thr Ser Ser Val Asn Lys His Phe Asp Gly Asn				
		430	435	440	
20	ggc gtt atc gct agc atg gag cac tgt aat gat ttc atg ttc ctt gga				1513
	Gly Val Ile Ala Ser Met Glu His Cys Asn Asp Phe Met Phe Leu Gly				
		445	450	455	460
	acc gaa gcc atc tct cta ggt cgt gtc ggt gat gac ttt tgg tgc acg				1561
25	Thr Glu Ala Ile Ser Leu Gly Arg Val Gly Asp Asp Phe Trp Cys Thr				
		465	470	475	
	gat cca tca ggc gac ata aac ggc aca tat tgg ctg caa gga tgc cac				1609
	Asp Pro Ser Gly Asp Ile Asn Gly Thr Tyr Trp Leu Gln Gly Cys His				
30		480	485	490	
	atg gtc cac tgt gcc tac aac agt ctt tgg atg gga aat ttc atc cag				1657
	Met Val His Cys Ala Tyr Asn Ser Leu Trp Met Gly Asn Phe Ile Gln				
		495	500	505	
35	cct gat tgg gac atg ttt cag tcc aca cat cct tgt gct gag ttc cat				1705
	Pro Asp Trp Asp Met Phe Gln Ser Thr His Pro Cys Ala Glu Phe His				
		510	515	520	
40	gct gct tct cgt gcc atc tcc ggt ggg ccc att tac atc agc gat tgt				1753
	Ala Ala Ser Arg Ala Ile Ser Gly Gly Pro Ile Tyr Ile Ser Asp Cys				
		525	530	535	540
	gtg ggc cag cac gat ttc gat ctc ttg aag cga ctc gtc ttg cct gac				1801
45	Val Gly Gln His Asp Phe Asp Leu Leu Lys Arg Leu Val Leu Pro Asp				
		545	550	555	
	ggt tcg att ttg agg tgt gag cac tat gca ctc cca act cgt gac cgt				1849
	Gly Ser Ile Leu Arg Cys Glu His Tyr Ala Leu Pro Thr Arg Asp Arg				
50		560	565	570	
	ctc ttt gaa gac cct ctt cat gat ggc aaa acc atg ctc aag att tgg				1897
	Leu Phe Glu Asp Pro Leu His Asp Gly Lys Thr Met Leu Lys Ile Trp				

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	aac ttg aac aag tac act gga att att gga gca ttc aac tgc caa gga			1945
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	590	595	600	
	gga gga tgg tgc aga gaa acc cga cgc aac caa tgc ttc tcc caa tgc			1993
	Gly Gly Trp Cys Arg Glu Thr Arg Arg Asn Gln Cys Phe Ser Gln Cys			
10	605	610	615	620
	gtt aac acg tta acc gcc aca aca aat cct aag gac gtt gaa tgg aac			2041
	Val Asn Thr Leu Thr Ala Thr Thr Asn Pro Lys Asp Val Glu Trp Asn			
	625	630	635	
15	agt ggg aac aac cca atc tcc gtt gaa aac gtt gaa gag ttt gct ttg			2089
	Ser Gly Asn Asn Pro Ile Ser Val Glu Asn Val Glu Glu Phe Ala Leu			
	640	645	650	
20	ttc ttg tct cag tct aag aag ctt gtg ttg tct gga cca aac gat gat			2137
	Phe Leu Ser Gln Ser Lys Lys Leu Val Leu Ser Gly Pro Asn Asp Asp			
	655	660	665	
	ctc gag atc act ttg gag cct ttc aag ttt gag cta atc act gtc tca			2185
25	Leu Glu Ile Thr Leu Glu Pro Phe Lys Phe Glu Leu Ile Thr Val Ser			
	670	675	680	
	cca gtt gtc act att gag ggt agt tcg gtt cag ttt gct cca atc gga			2233
	Pro Val Val Thr Ile Glu Gly Ser Ser Val Gln Phe Ala Pro Ile Gly			
30	685	690	695	700
	ttg gtt aac atg cta aac act agc ggt gca att cga tcc ttg gtg tat			2281
	Leu Val Asn Met Leu Asn Thr Ser Gly Ala Ile Arg Ser Leu Val Tyr			
	705	710	715	
35	cat gag gaa tcc gtt gag att gga gtt cgt ggt gct gga gag ttc agg			2329
	His Glu Glu Ser Val Glu Ile Gly Val Arg Gly Ala Gly Glu Phe Arg			
	720	725	730	
	gtt tat gca tca agg aaa cct gcg agc tgc aaa att gat ggt gaa gtt			2377
40	Val Tyr Ala Ser Arg Lys Pro Ala Ser Cys Lys Ile Asp Gly Glu Val			
	735	740	745	
	gtt gag ttt gga tac gaa gag tca atg gtg atg gtt caa gtg cct tgg			2425
	Val Glu Phe Gly Tyr Glu Glu Ser Met Val Met Val Gln Val Pro Trp			
45	750	755	760	
	tct gca ccc gag ggt ttg tct tct att aag tat gag ttt tag agtttccga			2476
	Ser Ala Pro Glu Gly Leu Ser Ser Ile Lys Tyr Glu Phe			
50	765	770	775	
	aggtgcttat ttgtatcctt cttaactcct taattatgag ctccgtgccg tttctttttc			2536
	tatatggttt ctgagagtga acatctaata ttaccact aggtataat tattggcttt			2596

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Gly Val Lys Cys Leu Val Asp Gly Gly Cys Pro Pro Gly Leu Val Leu  
35 40 45

Ile Asp Asp Gly Trp Gln Ser Ile Gly His Asp Ser Asp Gly Ile Asp  
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Val Glu Gly Met Ser Cys Thr Val Ala Gly Glu Gln Met Pro Cys Arg  
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Leu Pro Lys Phe Gln Glu Asn Phe Lys Phe Arg Asp Tyr Val Ser Pro  
85 90 95

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Lys Asp Lys Asn Glu Val Gly Met Lys Ala Phe Val Arg Asp Leu Lys  
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115 120 125

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Gly Tyr Trp Gly Gly Leu Arg Pro Gly Ala Pro Thr Leu Pro Pro Ser  
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Asp Leu Ala Val Asp Lys Ile Ile Asp Thr Gly Ile Gly Phe Val Ser  
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Phe Lys Ala Leu Thr Ser Ser Val Asn Lys His Phe Asp Gly Asn Ala  
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25 acg tgg gat gcg ttt tat ttg acg gtg aac cct gac gga gtt cat aag 96  
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35 atc gac gac ggt tgg caa tcg att gga cat gac tcc gat ggt atc gat 192  
Ile Asp Asp Gly Trp Gln Ser Ile Gly His Asp Ser Asp Gly Ile Asp  
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40 gtt gaa ggg atg agt tgt acc gtc gcc ggg gag caa atg cct tgc agg 240  
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45 ctt ccg aaa ttt caa gag aac ttc aag ttc aga gac tac gtc tct ccg 288  
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<213>	Artificial Sequence

15 <220>  
<223> Designed oligonucleotide primer to obtain raffinose synthase gene.

20 <400> 47  
aacgagctca atccaaaatc tcatcaaata atcgc 35

25      <210> 48  
         <211> 25  
         <212> DNA  
         <213> Artificial Sequence

30 <220>  
<223> Designed oligonucleotide primer to obtain raffinose synthase gene.

35      <400> 248  
acaatagttg agggcggaag agtag      25

40

$\langle 210 \rangle$	49
$\langle 211 \rangle$	25
$\langle 212 \rangle$	DNA
$\langle 213 \rangle$	Artificial Sequence

45 <220>  
<223> Designed oligonucleotide linker to obtain raffinose synthase gene.

<400> 49  
50 gatcgagctc gtgtcggatc cagct 25

<210> 50

55

<211> 17  
 <212> DNA  
 5 <213> Artificial Sequence  
  
 <220>  
 10 <223> Designed oligonucleotide linker to obtain raffinose synthase gene.  
  
 <400> 50  
 ggatccgaca cgagctc 17  
 15  
 <210> 51  
 <211> 30  
 <212> DNA  
 20 <213> Artificial Sequence  
  
 <220>  
 25 <223> Designed oligonucleotide primer to confirm direction of the inserted  
 raffinose synthase gene.  
  
 <400> 51  
 30 cctcctcgga ttccattgcc cagctatctg 30  
  
 <210> 52  
 <211> 30  
 35 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 40 <223> Designed oligonucleotide primer to confirm direction of the inserted  
 raffinose synthase gene.  
  
 <400> 52  
 45 ggattcgaca caaaccgcca cgtcatcgtc 30  
  
 <210> 53  
 50 <211> 29  
 <212> DNA  
 <213> Artificial Sequence  
 55

<220>

<223> Designed oligonucleotide primer to confirm direction of the inserted  
raffinose synthase gene.

<400> 53

tctacgtctg gcacgcgctt tgcggctac

29

# Claims

1. A raffinose synthase gene which comprises a nucleotide sequence hybridizable with a nucleotide sequence selected from the group consisting of:

- (a) a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 1,
  - (b) the nucleotide sequence represented by SEQ ID NO: 2,
  - (c) a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 3,
  - (d) the nucleotide sequence represented by the 236th to 2584th nucleotides in the nucleotide sequence represented by SEQ ID NO: 4,
  - (e) a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 5,
  - (f) the nucleotide sequence represented by the 134th to 2467th nucleotides in the nucleotide sequence represented by SEQ ID NO: 6,
  - (g) a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 7, and
  - (h) the nucleotide sequence represented by the 1st to 1719th nucleotides in the nucleotide sequence represented by SEQ ID NO: 8,
- under stringent conditions, and encoding a protein being capable of binding D-galactosyl group through  $\alpha$  (1 $\rightarrow$ 6) bond to the hydroxyl group attached to the carbon atom at 6-position of the D-glucose residue in a sucrose molecule to form raffinose.

2. A raffinose synthase gene comprising a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 1.

3. A raffinose synthase gene comprising the nucleotide sequence represented by SEQ ID NO: 2.

4. A raffinose synthase gene comprising a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 3.

5. A raffinose synthase gene comprising the nucleotide sequence represented by the 236th to 2584th nucleotides in the nucleotide sequence represented by SEQ ID NO: 4.

6. A raffinose synthase gene comprising a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 5.

7. A raffinose synthase gene comprising the nucleotide sequence represented by the 134th to 2467th nucleotides in the nucleotide sequence represented by SEQ ID NO: 6.

8. A raffinose synthase gene comprising a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 7.

9. A raffinose synthase gene comprising the nucleotide sequence represented by the 1st to 1719th nucleotides in the nucleotide sequence represented by SEQ ID NO: 8.

10. A raffinose synthase gene comprising the nucleotide sequence represented by SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8.
- 5 11. A nucleic acid comprising a partial nucleotide sequence of the raffinose synthase gene of any one of claims 1 to 10.
12. A method for detecting a nucleic acid containing a raffinose synthase gene which comprises detecting said nucleic acid by hybridization using the labeled nucleic acid of claim 11 as a probe.
- 10 13. A method for amplifying a nucleic acid containing a raffinose synthase gene which comprises amplifying said nucleic acid by polymerase chain reaction (PCR) using the nucleic acid of claim 11 as a primer.
14. A method for obtaining a raffinose synthase gene which comprises the steps of:
- 15 detecting a nucleic acid containing said raffinose synthase gene by hybridization using the labeled nucleic acid of claim 11 as a probe, and recovering the detected nucleic acid.
15. A method for obtaining a raffinose synthase gene which comprises the steps of:
- 20 amplifying a nucleic acid containing said raffinose synthase gene by PCR using the nucleic acid of claim 11 as a primer, and recovering the amplified nucleic acid.
- 25 16. A nucleic acid comprising a nucleic acid containing the raffinose synthase gene of any one of claims 1 to 10 which is joined to a nucleic acid exhibiting promoter activity in a host cell.
17. A vector comprising the raffinose synthase gene of any one of claims 1 to 10.
18. A transformant, wherein the raffinose synthase gene of any one of claims 1 to 10 is introduced into a host cell.
- 30 19. A transformant, wherein the nucleic acid of claim 16 is introduced into a host cell.
20. A transformant, wherein the vector of claim 17 is introduced into a host cell.
- 35 21. The transformant of any one of claims 18 to 20, wherein the host is a microorganism.
22. The transformant of any one of claims 18 to 20, wherein the host is a plant.
23. A method for producing a raffinose synthase which comprises the steps of:
- 40 culturing or growing the transformant of any one of claims 18 to 22 to produce the raffinose synthase, and collecting the raffinose synthase.
24. A raffinose synthase comprising the amino acid sequence represented by SEQ ID NO: 1.
- 45 25. A raffinose synthase comprising the amino acid sequence represented by SEQ ID NO: 3.
26. A raffinose synthase comprising the amino acid sequence represented by SEQ ID NO: 5.
- 50 27. A raffinose synthase comprising the amino acid sequence represented by SEQ ID NO: 7.